VITRIFICATION AND CHORIOALLANTOIC MEMBRANE (CAM) CULTURE OF BOVINE OVARIAN TISSUE

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the degree of Master of Science
in the Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon, Saskatchewan, Canada

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ABSTRACT

The overall objectives of this thesis were to develop a short-term culture system and to examine the effects of vitrification and short-term culture on the viability of fresh and vitrified bovine ovarian tissue and the follicles within.

The first objective was to compare the health and development of preantral follicles in bovine ovarian tissue, as well as the neovascularization of these tissues, subjected to avian chorioallantoic membrane (CAM) culture with the traditional in vitro culture system. We hypothesized that the chorioallantoic membrane (CAM) of the chicken embryo is a more suitable culture system than traditional in vitro culture. Bovine ovaries were retrieved from a local abattoir and cortical pieces (1-2mm^3) were randomly assigned to one of the following groups; control (fixed immediately), CAM or in in vitro culture. Ovarian tissue fragments from both groups were removed on D1, D3 and D5 of culture, fixed, sectioned (5μm) and stained with H&E. The numbers of healthy and degenerated follicles, primordial and activated preantral (primary and secondary), and the number of infiltrated bovine and avian blood vessels were determined using standard stereological procedures. All grafts placed on the traumatized CAM demonstrated increased neovascularization over time. The healthy primordial follicle density decreased over time concomitant with an increase in degenerated (primordial and activated preantral) follicles in both treatment groups. Healthy activated preantral follicle density did not differ between the two culture systems at a given time. In CAM group, blood vessel density increased over time ($p = 0.015$).

The second objective of this thesis was to develop a suitable vitrification protocol for bovine ovarian tissue. The viability of bovine ovarian tissue vitrified using two non-permeating cryoprotectants (sucrose and trehalose) and two cryodevices (cryotop and cryovial) was assessed. We hypothesized that during vitrification the higher cooling rate on the cryotop (open vitrification method) will yield better post-thaw viability of bovine ovarian tissue as compared to the cryovial (closed vitrification method). We also hypothesized that trehalose is a superior non-permeating cryoprotectant to sucrose for vitrification of bovine ovarian tissue. The ovarian tissue was fragmented (1-2mm^3) and divided into 6 different treatment groups. Tissues were vitrified in TCM199 supplemented with 15% EG, 15% DMSO, 20% calf serum and 0.5M sucrose or trehalose then placed in a cryovial or on a cryotop. After warming, the vitrified tissues were either immediately placed in 10% formalin (control) or on the chorioallantoic membrane of a 10-day old chicken embryo for 5 days. Follicles from control and vitrified tissue were observed under a light microscope for normal morphology and the total, normal and degenerated follicle densities were determined by standard stereological procedures. Sucrose and trehalose did not differ, nor was a difference observed between the cryovial and the cryotop for total, healthy or degenerated follicle density. Proportion of healthy follicles was higher in the control than all treatment tissues grafted to the CAM. All grafts placed on the traumatized CAM demonstrated presence of avian erythrocytes in the blood vessels after 5 days, but no difference was observed for blood vessel density among treatments. Lastly, the cooling rate of bovine ovarian tissue subjected to open and closed system devices for vitrification was evaluated. A thermocouple

wire was used to determine the cooling velocity of 1-2mm^3 fragments of bovine ovarian tissue placed on a cryotop (open system) or in a sealed cryovial (closed system). The cooling rate of tissues on the cryotop and in the cryovial was 7481±205.9° C/min and 664±26.0° C/min respectively.

In conclusion, the CAM supported the bovine ovarian tissue, thus the CAM culture system may be considered an acceptable alternative to traditional in vitro culture system for bovine ovarian tissue. Furthermore, angiogenesis may be an additional indication of ovarian tissue health. The hypotheses of our second study were refuted. Results indicated that sucrose and trehalose, and the cryotop and cryovial were equally effective in vitrifying bovine ovarian tissue.

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my supervisor, Dr. Muhammad Anzar for all of his patience, guidance, knowledge and support throughout my program. I would also like to thank my committee members, Drs. Jaswant Singh, Ali Honaramooz and Pritpal Malhi for their helpful input and insightful comments.

I am truly grateful to Agriculture and Agri-Food Canada and the Canadian Animal Genetic Resource Program for funding my project. I would also like to acknowledge the team at Drake Meats for supplying the ovaries for my projects, as well Robert Gonda and the U of S Department of Animal Science for supplying the eggs.

Appreciation is extended to all my mentors and colleagues in the One Health Reproductive group for providing a welcome working environment. To my fellow graduate students and office mates: Thank you for all of your help and support and for all of the good times we've shared in and out of the lab. This journey would not have been possible without friends to laugh with (and vent to). Thank you for being my home away from home.

I am so thankful to my best friend and boyfriend, Quinton Rook, for consistently encouraging me on this journey, even at a distance. Thank you for your unwavering love and support and for truly making me a better person.

Lastly, I thank my friends and family at home in Québec. You have taught me the value of working hard to achieve a goal and I know that I could not have finished this thesis without all of you. To my parents Les and Jackie and my younger siblings, Kellie Ann, Keri and Konnor: Thank you for inspiring and motivating me when I needed it the most; I dedicate this thesis to you.

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LIST OF ABBREVIATIONS

µl: Microliter

µm: micron

°C: Degrees Celsius

AMH: Anti Müllerian hormone

Angpt-2: angiopoetin-2

AVG: Average

CAM: Chorioallantoic membrane

cm: centimeter

CO_2: Carbon Dioxide

CP: Cryoprotectant

CS: Newborn calf serum

D: Day

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPBS: Dulbecco's phosphate buffer saline

EG: Ethylene glycol

FAO: Food and Agriculture Organization of the United Nations

GV: Germinal vesicle

h: hour

H_2O_2: Hydrogen Peroxide

ITS: Insulin-transferrin-selenium

IVF: In vitro fertilization

LN_2: Liquid nitrogen

M: Molar

MII: Metaphase II

mIU: Milli- international units

ml: Milliliter

mm: millimeter

mRNA: Messenger ribonucleic acid

n: Number

OTC: Ovarian tissue cryosystem

PBS: Phosphate buffer saline

rFSH: Recombinant follicle stimulating hormone

SSV: Solid-surface vitrification

TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

v/v: volume for volume

VEGF: Vascular endothelial growth factor

VS1: Vitrification solution 1 (equilibrium solution)

VS2: Vitrification solution 2

CHAPTER 1: INTRODUCTION

Many domestic breeds of livestock are facing a gradual diminishment of genetic diversity due to selection pressures and environmental factors. It is estimated that approximately 20% of the world's breeds of cattle, goats, pigs, horses and poultry are currently at risk of extinction and in the last decade, one breed per month has been lost forever. The current loss of genetic resources concerns not only the extinction of traditional breeds, but also the loss of genetic diversity within breeds (Taberlet *et al.* 2011). Without immediate action within a few decades, we may lose most of the highly valuable farm animal genetic resources that humanity has gradually selected over thousands of years (Taberlet *et al.* 2008).

Increased awareness of depleting animal genetics diversity has prompted national and international efforts to restore the at-risk breeds. Ideally, populations of live animals could be protected, however this practice is a very slow and expensive process and is not likely to succeed (Patterson and Silversides 2003). Therefore the in vitro collection of germplasm and the creation of a global gene bank have been proposed as an effective alternative for the conservation of animal genetic resources. Conservation efforts have been focused on the cryopreservation of gametes, embryos, gonadal tissue and somatic cells. While there has been considerable success for the preservation of semen and embryos (particularly in cattle), limited achievements have been observed for the cryopreservation of gonadal tissues. The large pool of immature gametes within gonadal tissues maximizes the genetic potential of an individual, making this technique one of interest and high value.

As of yet, there is no universal protocol for the cryopreservation of gonadal tissues. Given the variety of cell types within the tissue, there are an infinite number of variables that need to be considered. The permeability, toxicity and exposure time of cryoprotectants as well as the size and density of the tissues and the rates of cooling and warming should be taken into account.

Intracellular ice formation and osmotic injury are the leading causes of tissue and follicle damage upon cryopreservation. The most effective way to avoid such complications is to rapidly cool and warm tissues. Vitrification, a method in which cells are exposed to high concentration of cryoprotectants and frozen at an ultra-rapid cooling rate, reduces the likelihood of intracellular ice formation by transforming the highly viscous intracellular compartment into a vitreous (glass-like) state. Unlike slow freezing, in which a cell-specific cooling rate is used, vitrification is not a cell-specific procedure and this is more favorable for the cryopreservation of multicellular tissues.

Concerning ovarian tissue specifically, immature follicles (such as primordial and activated preantral) within the cortex are relatively more resistant to cryopreservation than their more mature counterparts (Gosden *et al.* 2002). These follicles are smaller in diameter, have fewer organelles and are more apt to repair sub-lethal damages incurred during the cryopreservation process (Shaw *et al.* 2000).

One of the challenges in preserving immature follicles is their ability to grow after warming and develop to a mature state (Comizzoli and Wildt 2014). In vitro growth of preantral follicles resulting in live births has only been successful in the mouse model (Eppig and O'Brien 1996; O'Brien *et al.* 2003). In vivo culture (xenografting) provides an alternative to traditional in vitro culture. The grafting of ovarian tissue to an

immunodeficient species, such as the nude or SCID mouse, to the chorioallantoic membrane (CAM) of the avian embryos or back to the individual of origin (autografting) have proven to be effective methods in growing the immature oocytes stored in ovarian tissue. One of the crucial steps in the functional restoration of these grafts is revascularization and the prevention of ischemia and hypoxia. It has been shown that decreased follicular density in the grafted ovary is more related to ischemic factors rather than to cryoinjury (Nisolle *et al.* 2000), therefore studies of angiogenesis and neovascularization are of priority.

The objectives of this thesis were to develop a short-term culture system and to examine the effects of vitrification and short-term culture on the viability of fresh and vitrified bovine ovarian tissue and the follicles within.

CHAPTER 2: LITERATURE REVIEW

2.1 CONSERVATION OF ANIMAL GENETIC RESOURCES

2.1.1 Loss of farm animal genetic resources

Livestock plays a significant role in agricultural, economic, cultural and religious sectors in both early and current society (Li *et al.* 2012). The livestock species contributing to today's agriculture and food production are shaped by a long history of domestication and development (Pilling and Rischkowsky 2007). In the past century, change and selection pressure by environmental factors and human intervention have caused a loss in genetic diversity and an increased rate of extinction of many rare livestock breeds. The industrialization of agriculture and expansion of large-scale production has resulted in a higher demand for meat milk and eggs. To meet the market's demand, higher yielding breeds, or lines within breeds, are selected. Therefore the market is dominated by a small number of breeds and the remaining breeds become endangered.

Current reproductive technologies have also contributed to the loss of livestock diversity. A single individual can produce a large number of progeny and modern transportation systems have enabled the rapid and efficient distribution of germplasm around the world. Breeding programs carried out by national and international companies place intense selection pressure on few breeds and few superior individuals within the breed which further deplete genetic diversity (Patterson and Silversides 2003).

According to the Food and Agriculture Organization (FAO) (Pilling and Rischkowsky 2007), approximately 20% of the world's breeds of cattle, goats, pigs, horses and poultry are currently at risk of extinction. At least one livestock breed has become extinct per month

over the past several years, resulting in the loss of genetic characteristics forever (Buerkle 2007). In Canada, there are fifteen breeds of cattle on the Rare Breeds Canada Conservation List (RBC 2014) Table 2.1 organizes these breeds according to their risk status.

Table 2.1. Risk status of cattle breeds, Adapted from Canada's Livestock Conservation List 2014

Status (Annual Registration of 100% pure female stock)	Breed
Critical (1-25)	Lynch Lineback, White Park, Texas Longhorn
Endangered (26-75)	Belted Galloway, Hays Converter, Kerry, Lincoln Red, Milking Shorthorn, Red Poll
Vulnerable (76-250)	Braunvieh, Canadienne, Dexter, Galloway, Geurnsey, Highland
At Risk (251-750)	N/A

Rare Breeds Canada (RBC 2014)

The extinction of a breed or population disrupts the function of the entire global ecosystem and leads to the loss of the breed's unique adaptive qualities. It is particularly important to conserve these rare and endangered breeds because their ancestors no longer exist (Long 2008) and it is essential that strenuous efforts to understand and protect the world's animal genetic resources be put in place so that their potential can be evaluated and conserved.

2.1.2 Animal genetic conservation strategies

Increased awareness of depleting animal genetic diversity has prompted global and national efforts for the conservation of threatened breeds. Organizations such as Rare Breeds Canada promote genetic diversity for breed security by informing Canadians on the agricultural heritage of rare livestock breeds through education and niche marketing (RBC

2014). Government initiatives such as the Canadian Animal Genetic Resources Program (CAGR) have also focused their efforts on ensuring genetic diversity of Canadian livestock, supporting environmentally friendly livestock and poultry production and maintaining food security by acquiring, evaluating and cryopreserving tissue and germplasm (AAFC 2013). Maintaining genetic diversity also provides insurance against climate and social change, disease, selection errors and unforeseen catastrophe (Patterson and Silversides 2003).

Conservation of farm animal genetic resources has become important not only for their biological role in maintaining biodiversity, but also for their economic, social and cultural values (Barker 2001). The goals of conservation are to keep genetic variation as gene combinations in a reversible form and to keep specific genes of interest (Prentice and Anzar 2010).

Different strategies have been implemented to help preserve rare breeds. *In situ* conservation is the maintenance of a breed within a livestock species by enhancing their productive potential in their natural environment. As an alternative, *ex situ* conservation can be applied to establish a viable population by the application of reproductive technologies. *Ex situ in vivo* conservation is the safeguarding of live animals in small populations (such as parks, zoos and sanctuaries), with the hopes of propagating the breed in a natural way. This system is not practical however because, for large breeds of livestock, the gestation time and interval between generations is very long therefore making breed regeneration a time-consuming process. But, such a small population does not allow for considerable genetic diversity within the breed which, due to inbreeding, may lead to poor

adaptive capacity and the risks of transmission of inherited diseases, congenital defects and fertility problems (Comizzoli *et al.* 2000).

Lastly, *ex situ in vitro* conservation is the preservation of animal genetic resources in haploid (semen and oocytes) or diploid (embryos) form or as DNA sequences. These conservation programs accelerate genetic progress, reduce the risk of disease transmission and expand the number of animals that can be bred from the superior parent (Pilling and Rischkowsky 2007). The banking of gametes and genetic material is not only essential for reestablishing rare breeds, as it would allow more offspring to be obtained from selected parents, ensuring genetic diversity, but also has great potential for biomedical research, treatment of human infertility and preserving important and useful strains of laboratory animals (Agca 2000).

2.1.3 Gametes and Embryos

Artificial insemination has become one of the most widely used techniques in livestock production. It is estimated that a bovine breed could be saved with 1000 semen doses from 25 individuals (Comizzoli *et al.* 2000). Although semen is abundant and highly accessible it contributes only one half of an animal's genetic material. A concern in terms of semen banking for conservation purposes is the number of doses required for storage. According to the FAO, the number of doses required to regenerate a population or a breed is a function of the number of doses required per parturition (conception rate), the expected lifetime production index (LPI) of the founder females, and the number of males and females required in the reconstructed population. Through back-crossing, it could be

possible to reconstruct the nuclear genome of an “at risk” species, but the efficiency and speed of such method depends on the length of reproductive cycles and gestation as well as the conception rate (Mara *et al.* 2013). Semen from most livestock and a few avian species has ben successfully cryopreserved in recent years (Pilling and Rischkowsky 2007). The cryopreservation of semen is well established in the bovine species however the full diversity cannot be established because semen from only superior males is collected.

The cryopreservation of the other half of genetics, the female germline, is equally as important for breed propagation. The success of cryopreservation of oocytes is inconsistent and the challenge may be due to an oocytes variety of cell stages (germinal vesicle; GV, and metaphase II; MII), the large size of the cell, and little capacity to repair damages (Lucci *et al.* 2004). Additionally, the oocyte has a low surface to volume ratio, making dehydration and cryoprotectant penetration difficult to achieve (Prentice and Anzar 2010). Concern has also been raised about the disruption of the meiotic spindle in MII oocytes as they undergo cooling. At low temperatures, the tubulin in the meiotic spindle depolymerizes, disassembling the spindle fibers causing improper alignment and segregation of chromosomes during the maturation process (Zenzes *et al.* 2001; Mandelbaum *et al.* 2004), Also, the stress of cryopreservation can cause the premature release of cortical granules and subsequent hardening of the zona pellucida (Gosden 2000), blocking sperm penetration. Despite the many advances in oocyte cryopreservation, an ideal procedure has yet to be established and thus it is still considered an experimental technique (Noyes *et al.* 2010).

The cryopreservation of embryos allows the conservation of the full genetic complement of both dam and sire. Complete reconstruction of a species or breed requires a

very high number of donor females. In the case of some critically endangered breeds, it may be difficult to obtain so many embryos. For breed or species reconstruction it is projected that the collection and storage of 300 unsexed embryos from 90 donors would be needed. Multiple embryo collections would be required from each superovulated donor female. In the case of cattle however, superstimulatory response and subsequent embryo recovery is variable (Mapletoft *et al.* 2002).

The techniques used in embryo collection and embryo freezing are continually improving, but cryopreservation of embryos is still more complex, difficult and costly than the cryopreservation of semen (Boettcher *et al.* 2005). Embryos from most mammalian species have been successfully cryopreserved. The embryos of some species, such as swine, are more cryosensitive than bovine and ovine, therefore the current use of cryopreservation from a commercial standpoint is limited to these ruminants (Niemann and Rath 2001).

2.1.4 Reproductive Tissue

The successful cryopreservation of gonadal tissue provides a powerful tool to maximize genetic potential by preserving the pool of immature germ cells. These cells can later be cultured in vitro or grafted to a host and grown to produce mature cells for IVF. Such practices may change the reproductive management of populations of species (Comizzoli and Wildt 2014). Effective growth and differentiation of germ cells from young, pre-pubertal animals would allow for faster genetic propagation (especially in animals with a late puberty and/or a long gestation time). Due to the complexity and heterogeneity of

cells types within the tissue however, substantial research is required to streamline this technique for commercial use.

An alternative strategy for conserving female gametes is the cryopreservation of ovarian tissue. This technique allows the storage of a large number of primordial follicles (especially in young animals) in the ovarian cortex, which tend to be more tolerable to freezing (Jewgenow *et al.* 2011). The oocytes in primordial follicles are less likely to be damaged because they do not yet have a zona pellucida, several layers of cumulus cells or peripheral cortical granules, are smaller in size, have fewer organelles than more mature oocytes, have a low metabolic rate and few intracytoplasmic lipids (which are cold sensitive) (Lucci *et al.* 2004). Also, the oocytes in ovarian tissue are arrested in the prophase stage and theoretically have a lower risk of enduring genetic-altering damage upon cryopreservation (Shaw *et al.* 2000).

There has also been a rising interest in the cryopreservation of testicular tissue. As with ovarian tissue, testicular tissue contains an abundant source of germ cells at varying developmental stages (Picton *et al.* 2000). While mature sperm are routinely cryopreserved, and small immature germ cells (when isolated and placed in cell suspension) can be successfully cryopreserved (Avarbock *et al.* 1996; Brinster and Nagano 1998; Izadyar *et al.* 2002), challenges are still present in freezing testicular tissue. In humans there has been some success in cryopreserving testicular biopsies and extracting mature spermatozoa upon thawing for intracytoplasmic sperm injection (ICSI) (Salzbrunn *et al.* 1996). Among domestic species, testes tissue of the pig (Honaramooz *et al.* 2002; Zeng *et al.* 2009; Abrishami *et al.* 2010; Yang *et al.* 2010) goat (Honaramooz *et al.* 2002), cattle

(Wu *et al.* 2011) and chicken (Song and Silversides 2007b) have been cryopreserved using a variety of cryoprotectants.

2.2 OVARIAN TISSUE CRYOPRESERVATION

2.2.1 History

Cryopreservation of ovarian tissue has been widely applied as a promising alternative technique for preserving female fertility and genetic resources. The first report of ovarian tissue cryopreservation was in the mid 1950s (Parkes and Smith 1953; Deanesly 1954; Parkes 1956). Ovarian tissue grafts from mice were subjected to glycerol, then placed at -79°C. The rate of cooling was not controlled in this study and the authors reported a follicle survival rate of 5% after freezing. Later, Candy *et al.* (1995) reported 20% follicle survival rate when the cooling velocity was controlled. Since then, with the use of more effective methods and cryoprotectants, success has been achieved with slow freezing (Agca 2000; Courbiere *et al.* 2005; Baudot *et al.* 2007) as well as with vitrification (Courbiere *et al.* 2006; Santos *et al.* 2007; Amorim *et al.* 2011), but the results of the latter are discrepant both among species and within species. In more recent publications, vitrification has been shown to have equal and even superior results over slow freezing (Song *et al.* 2007), but further studies are required to perfect this technique.

Cryopreservation protocols for ovarian tissue have been greatly improved in the last 30 years and are largely based on embryo freezing protocols (Mara *et al.* 2013). Transplantation of frozen-thawed ovarian tissue in the lamb (Baird *et al.* 2004), non-

human primate (Lee *et al.* 2004) and human (Donnez *et al.* 2004) have resulted in live births, giving optimism that the same can be achieved in other species.

2.2.2 Human Ovarian Tissue Cryopreservation

Ovarian tissue cryopreservation is an ideal method of fertility preservation for adult women who do not respond to ovarian stimulation or when embryo freezing is not feasible or desired (Rodriguez-Wallberg and Oktay 2012). In the case of cancer patients, ovarian tissue cryopreservation is the only method for young girls for preserving their fertility after treatment (Chemotherapy, radiation etc.). Chemical and radiation therapies are gonadotoxic; therefore the collection of tissue prior to treatment and their cryopreservation, thawing and transplantation back to the patient may restore fertility during remission. This technology is advantageous as a patient can have restoration of both endocrine and fertility function. For cancer survivors, the risk of re-implantation of aggressive cancer cells is still a serious issue. Some cancers have an elevated metastatic spread to the ovary therefore additional methods for use of stored ovarian tissue are needed to minimize the risk of cancer potentiation (Smitz *et al.* 2010). Cryopreservation of human ovarian tissue differs in this respect to that of livestock species, which will be used for post-thaw oocyte retrieval instead of re-implantation (Jewgenow *et al.* 2011). The first reported method of human ovarian tissue cryopreservation was in 1996 (Hovatta *et al.* 1996). Donnez *et al.* (2004) reported the first live birth after autotransplantation of cryopreserved ovarian tissue and since then, this technique has lead to the birth of 17 healthy babies (Silber *et al.* 2010; Donnez and Dolmans 2011; Revel *et al.* 2011).

2.2.3 Principles

Cryopreservation is a suitable practice for the conservation of genetic material in a cost-effective manner as it reduces the expenses, genetic drive and diseases that are associated with maintaining live animals and cell lines (Shaw *et al.* 2000). It is a multistep procedure that involves the exposure of cells and tissues to cryoprotectants, cooling to sub zero temperatures, storage in liquid nitrogen (conventionally; -196 °C) and thawing and removal of the cryoprotectants with the overall goal of maintaining cell and/or tissue integrity. Cryopreservation at such a low temperature effectively stops all biological activity due to the unavailability of water, maintaining the cell viability and functional state for centuries (Mazur 1970).

Unfortunately, there is no universal protocol for cryopreservation of different cell types because there are an infinite number of variables to be considered. Different cell types and even species-specific difference among cell types add to the complexity of the technique. It is important to consider the nature and concentration of cryoprotectants used. Moreover, the freezing method and cooling rate are important factors in minimizing cryoinjury of cells.

2.2.4 Cryoinjuries

The extent of cryodamage to cells depends on the size, shape and type of cell as well as the membrane permeability. These factors can be highly variable depending on species, developmental stage and origin (Vajta and Kuwayama 2006). Water is essential for most biological functions, but the damage it causes upon freezing (intracellular ice formation)

poses a challenge for successful cryopreservation. When water freezes within a cell, it expands and purifies, excluding biological solvents and creating pure sharp ice crystals (Wowk 2007). Upon thawing, these ice crystals may reform and grow (Seki *et al.* 2014). Tissues are more difficult to freeze than isolated cells. Unlike cell suspensions, there is little room for ice to expand within tissues, thus making them more likely to be damaged by ice crystal formation. Also, with the diverse cell types within a tissue, and each cell population having their own tolerance to freezing, satisfying the needs of all cell types is challenging (Kim 2006).

A detailed literature review of vitrification protocols of ovarian tissue in various species is summarized in Table 2.2.

2.2.5 Freezing Procedures

The two main cryopreservation techniques used for the preservation of biological samples are slow freezing and vitrification. Slow freezing is the gradual cooling of material subjected to low levels of cryoprotectant followed by immersion in liquid nitrogen. Ice first forms in the external medium, which causes an osmotic gradient between the external medium and the intracellular water, causing an osmotic drive for water to exit the cell and freeze externally (Seki *et al.* 2014). The rate of cooling is slow enough that the water balance between the cell and the external medium remains at equilibrium while the cell is super-cooled. Often, however, the rate of cooling in conventional slow freezing may not be fast enough and can lead to intracellular ice crystallization in some cryosensitive cell types

(Ledda *et al.* 2006). This method is also disadvantageous, as it requires a programmable freezer that may not be available under field conditions.

Vitrification on the other hand, is the rapid cooling of material subjected to very high concentrations of cryoprotectants. Vitrification reduces the damage associated with intracellular ice formation by transforming the inside of the cell from a crystalline liquid to a non-crystalline solid or "glass-like" state. For this reason, vitrification has been shown more recently to improve tissue cryopreservation, as every cell within tissue becomes glass like, regardless of cell type. In this procedure, the cells are exposed to approximately 5-7M concentration of CPs (Rall 1987; Massip *et al.* 1995). At such a high concentration, the cells can only be exposed to CPs for a short time with minimum volume to increase the cooling rate and reduce the risk of CP toxicity (Mara *et al.* 2013). The rapid removal of cryoprotectants has proven more difficult in sperm cells than in oocytes, embryos and tissues; therefore, vitrification is not recommended for the cryopreservation of semen (Jewgenow *et al.* 2011). Vitrification is also advantageous in that the exposure time to non-physiological conditions before storage is much lower than slow freezing (Campos-Chillon *et al.* 2006). Moreover, on a per pregnancy basis, vitrification is much cheaper than a stepwise freezing process (Campos-Chillon *et al.* 2006). It has been stated that vitrification of embryos is an integral part of IVF programs and can be exploited in a commercial setting (Menezo 2004). In the following section, different factors affecting the vitrification of tissue will be discussed.

Table 2.2: Vitrification system and cryoprotectants used in ovarian tissue vitrification in the past 15 years.

Species	Vitrification system (open/closed)	Cryodevice Used	Cryoprotectants Used (permeating, non-permeating, combination)	Reference
Mouse	Closed	Cryotube, Cryovial, plastic straw	Combination	Exp Anim 2000; 39:1: 17-21; Exp Anim 2002; 51(5): 509-12, Reproduction 2006; 131(4): 681-7; Mol Reprod Dev 2008; 75(4): 608-13; Lab Anim (NY) 2008; 37(8): 353-7; Mol Hum Reprod 2009; 15(3): 155-164; J Assist Reprod Genet 2009; 26(7): 415-20.
	Closed	Cryovial	Permeating	Biol Reprod 2003; 68(3): 881-7
	Open	Cryotop, cryovial (pour over), polyester sheet, SSV, EM Grid	Combination	Fertil Steril 2004; 81 (suppl 1); 824-830; Hum Reprod 2006; 21(11): 2794-800; Fertil Steril 2006; 86(Suppl 4): 1182-92; Cryobiology 2007; 54(1): 55-62; Cryobiology 2008; 57(2): 163-9; BMC Biotechnol 2008; 8: 38; Obstet Gynecol Sci 2013; 56 (6): 382-388
	Open	Cryotop	Permeating	Reprod Biomed Online 2007; 14(6): 693-9
Human	Closed		Combination	Fertil Steril 2006; 85(Suppl 1): 1150-6; BioMed Res Int 2014
	Closed	Cryovial	Permeating	Cryobiology 2007; 55(3): 261-8.
	Open	Copper Grid, Straw, Cryotop, Direct LN_2, Cryotissue, Cryostraw	Combination	Eur J Obstet Gynecol Reprod Biol 2003; 108(2): 186-93.; Reprod Biomed Online 2004; 9(2): 187-93; Reprod Biomed Online 2009; 18(4): 568-77, Hum Reprod 2009; 24(7): 1670-83; Cryobiology 2010; 60(2): 101- 5; Fertil Steril 2010; 94(6): 2191-2196; Reprod. Biomed. Online 2011; 23: 2: 160–186; Reprod Biomed Online 2014; Hum Reprod 2015;
	Open	SSV	Permeating	Hum Reprod 2008; 23(2): 336-9.
Hamster	Closed	Cryotube	Combination	Exp Anim 2000; 39:1: 17-21.
Rat	Closed	Cryotube	Combination	Exp Anim 2000; 39:1: 17-21.
	Open	Glass test tube	Combination	Theriogenology 2000; 53(5): 1093-103.
Rabbit	Closed	Cryotube	Combination	Exp Anim 2000; 39:1: 17-21.
Monkey	Closed	Cryotube, Plastic Straw	Combination	Exp Anim 2000; 39:1: 17-21; Hum Reprod 2013; 28(5): 1267-1279.
Dog	Closed	Cryotube	Combination	J Reprod Dev 2006; 52(2): 293-9.
Sheep	Closed	Cryotube, Cryobag, OTC	Combination	Hum Reprod 2005; 20(10): 2745-8; Fertil Steril 2006; 86: 1243–1251; Reprod Dom Anim 2014;
	Open	SSV	Combination	Anim Reprod Sci 2002; 71: 101-110; Reprod Dom Anim 2014
Cow	Closed	Plastic Straw	Combination	Fertil Steril 2006; 85(Suppl 1): 1150-6;
	Closed	Cryovial	Permeating	An Reprod Sci 125.1 (2011): 49-55.
	Open	Cryotop	Combination	Reprod Biomed Online 2009; 18(4): 568-77.
Pig	Closed	Plastic Straw	Combination	Fertil Steril 2006; 85(Suppl 1): 1150-6.
	Open	EM Grid, Cryotop	Combination	Cryobiology 2007; 54(1): 55-62; Theriogenology 2009; 72(2): 280-8.
Goat	Closed	Plastic Straw, OTC	Combination	Cell Tissue Res 2007; 327(1): 167-76; Anim Reprod Sci 2013; 138: 220-227; Reprod Fertil Dev 2014;

2.2.6 Cryoprotectants

Most tissues cannot survive sub-zero temperatures unless they are placed in protective solutions and are cooled and warmed at specific rates. To avoid ice formation and osmotic shock during freezing procedure cryoprotectants (CPs) are used. They are divided into two categories: permeating and non-permeating (Mara *et al.* 2013). Permeating CPs (such as propylene glycol, ethylene glycol, glycerol and dimethyl sulphoxide) infuse the cell membrane and bind to intracellular water molecules, thus lower the freezing temperature of the resulting mixture and also act to prevent excessive dehydration of the cells during the freezing process. The molecular mass of permeating CPs is less than 100 Daltons (Wowk 2007). Non-permeating CPs (such as sucrose, galactose, and trehalose) do not cross the cell membrane but increase the osmolality of the extracellular compartment and water diffuses out from the cells interior, causing cellular dehydration. In addition to being water-soluble, effective cryoprotectants depress the melting point of water, do not precipitate or form hydrates, and should be relative non-toxic to cells at high concentrations (Wowk 2007).

It has been suggested that toxicity is more related to the hydrogen bonds between water and the cryoprotectants rather than the concentration of cryoprotectants (Fahy *et al.* 2004). It is known that toxicity depends much more on permeating cryoprotectants than extracellular non-permeating CPs (Fahy *et al.* 1990). It is for this reason that non-permeating CPs are included in cryoprotectant media to increase the total concentration of CPs without drastically increasing the toxicity due to permeating CPs.

Using cryoprotectants in combination has been shown to improve post thaw viability of tissues. Lucci *et al.* (2004) reported that follicles frozen in 10% glycerol showed ultra structural damage such as damages to the organelles and disorganization of the cytoplasm structure of granulosa cells and oocytes, while follicles cryopreserved in dimethyl sulphoxide and propylene glycol exhibited normal ultrastructure.

In terms of non-permeating cryoprotectants, the morphological preservation of preantral follicles in rabbits increased in the presence of sucrose compared to trehalose (Neto *et al.* 2008). Also, while the number of viable follicles in the control sucrose group was lower than the trehalose control group, the proportion of morphologically normal follicles was higher in sucrose. Very recently, similar results were obtained in human ovarian tissue (Tian *et al.* 2015). There was no statistical difference between sucrose and trehalose treated groups but a trend towards trehalose suggested it may be a better sugar for primordial follicle preservation.

2.2.7 Cooling Rate

The success of vitrification is directly related to the cryoprotectants used and the cooling velocity of the tissue and is inversely related to the size of the tissue and volume of the surrounding medium. It has been reported that a cooling velocity of approximately 2500°C/min can achieve a vitrified state, (Palasz and Mapletoft 1996). However, the use of different suitable carrier systems may result in cooling rates approaching 20000°C/min (Vajta *et al.* 1998). By using ultra-rapid cooling rates, tissues pass though the critical temperature zone (between 15°C and -5°C) more quickly, which decreases chilling injury,

and allows for a lower concentration of cryoprotectants, decreasing potential toxic and osmotic damage (Orief *et al.* 2005).

Liquid nitrogen boils at -196°C. As tissues are immersed in LN_2, the LN_2 is warmed, inducing excessive boiling and release of nitrogen gas. As evaporation occurs a vapour layer surrounds the tissue, creating a layer of insulation, which decreases heat transfer, resulting in a decreased cooling rate (Orief *et al.* 2005). To lessen these effects, it is important to minimize the amount of solution surrounding the tissue upon freezing, as the extra solution creates a barrier for heat transfer. Another method is to prevent vapour formation by super cooling the liquid nitrogen. Applying a negative pressure to the LN_2 with a vacuum will transform the LN_2 to a slush state, decreasing its boiling temperature to -205°C or -210°C which will ultimately increase the cooling rate (Arav *et al.* 2000).

Cooling velocity, as well as warming rate, has been carefully measured with a device developed by Kleinhans *et al.* (2010), which is composed of an aqueous sample on a cryotop tip with a thermocouple wire.

2.2.8 Open System vs. Closed System

The possibility of contamination from liquid nitrogen possessing viral or bacterial pathogens is generally overlooked, but it is incorrect to assume it is sterile (Morris 2005). While vitrification allows rapid cooling of material, it may lead to the contamination of biological samples as a result of direct liquid nitrogen exposure (Vajta *et al.* 1998).

There are two kinds of cryodevices in which tissues can be frozen i.e. open containers in which there is a direct contact of the sample with liquid nitrogen or closed

containers with no direct contact. Open systems (such as the cryotop) offer the possibility of an increased cooling rate, however these systems have a risk of contamination and transmission of disease (Cutting *et al.* 2009). Efforts have been made to decrease or eliminate this risk by freezing tissues on a solid surface (Huang *et al.* 2008), or within a cryocontainer. Mirabet *et al.* (2012) noted that while contamination by liquid nitrogen exposure may occur on the outside of the container, it is much less likely that such contamination will affect the contents within. Bielanski *et al.* (2000) tested the effects of liquid nitrogen contaminated with bovine viral diarrhea virus (BVDV), bovine herpesvirus-1 (BHV) or bovine immunodeficiency virus (BIV) on the infection of vitrified bovine embryos. This study revealed that 21% of batches exposed to BVDV and BHV tested positive for viruses whereas none of the embryo batches exposed to BIV displayed infection.

Questions also arise about the possibility of contamination during storage of samples in liquid nitrogen. In a separate study Bielanski *et al.* (2003) reported on the microbial contamination of semen and embryos that had been stored in sealed plastic straws for 6-35 years. Although 32 strains of bacteria and 1 fungus were identified from samples of liquid nitrogen, they concluded that there was no risk of contamination from BVDV and BVH-1 in sealed plastic straws, or in the nitrogen itself. The proper handling of samples and freezing in aseptic liquid nitrogen technical concerns relating to hygiene and the health and safety of samples may be eliminated (Do *et al.* 2014).

2.2.9 Warming Procedures

While the freezing rate plays a critical role in maintaining cell integrity, the rate of thawing is equally as critical. It has been shown that a high warming rate is more crucial for cell survival than a high cooling rate in vitrification (Seki and Mazur 2009; Seki and Mazur 2012). In the same study (performed on mouse oocytes) it was discovered that as long as the warming rate was rapid enough, the concentration of CPs could be diluted by half without significantly affecting the oocytes. A high warming rate prevents the recrystallization of intracellular ice upon warming (Seki *et al.* 2014).

2.2.10 Quality assessment of frozen-warmed tissue

Histological evaluation has always been the most common approach to assess frozen-thawed and cultured follicles (Smitz *et al.* 2010). Parameters that can be assessed using histological analysis include: follicle density, identification of cell and follicle types and calculating the diameter of follicles. Immunohistochemistry has also proven very useful for assessing tissue health. Cell proliferation stains can assess functionality of the tissues and apoptosis can be evaluated by a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Hormone production in spent media (particularly estradiol) using a radioimmunoassay can be measured and follicular development can be evaluated at a molecular level by quantitative PCR and microarrays.

2.3 CULTURE OF OVARIAN TISSUE

Despite the growing interest in ovarian tissue banking, there are very few research initiatives on how to ensure the functionality of the stored tissue once thawed (Smitz *et al.* 2010). More recent advances in in vitro reproductive technologies have opened up new opportunities in biotechnology. The activation and development of a preantral follicle culture system that could potentially produce large quantities of oocytes would significantly advance the use of these techniques and make possible the preservation and long-term storage of the female germplasm (Gutierrez *et al.* 2000). The follicles (from carnivorous species) can be maintained in live culture for approximately 6 months to allow the recovery of viable oocytes (Songsasen *et al.* 2012).

The survival of the oocyte in the follicle depends on the health of the surrounding tissue and the ability of the granulosa cells to supply nutrients. The granulosa cells are also responsible for keeping the oocyte in meiotic arrest by gap junction signaling. Disruption of this junction would result in spontaneous breakdown of the germinal vesicle (Gutierrez *et al.* 2000).

2.3.1 In Vitro Culture

One of the most significant challenges in preserving early stage follicles within the ovary is the ability to initiate maturation and the capacity for fertilization in vitro (Comizzoli and Wildt 2014). Successful in vitro growth of preantral follicles has only been reported in the mouse model (O'Brien *et al.* 2003). The anatomical and physiological difference among larger livestock species, and in comparison to the mouse, makes it very

difficult to apply the same culture conditions and achieve similar results. Normal offspring have been born from newborn mice ovaries cultured in vitro for 8 days followed by enzymatic digestion of follicles and subsequent culture to a secondary follicle stage (Eppig and O'Brien 1996; O'Brien *et al.* 2003).

Unlike mice, in which the formation of primordial follicles occurs shortly after birth, folliculogenesis in most domestic species takes place during fetal life. This is advantageous in the mouse model as it allows for a uniform pool of follicles, however the same is not necessarily true in livestock species. Murine ovaries are also small enough to be cultured as intact whole ovaries and the ovarian stroma is not as dense permitting easier and less harmful follicle isolation by enzymatic digestion (Wandji *et al.* 1996).

To minimize the damaging effects of enzymatic digestion on bovine preantral follicles, pieces of cortical tissue were cultured in vitro to activate the primordial follicles within tissue. Fetal bovine ovaries were used because they are smaller, rich in primordial follicles and softer than those of adults (Braw-Tal and Yossefi 1997). The follicles were activated within the first 2 days of culture and was followed by follicular and oocyte growth. Although activation did occur, very few primary follicles developed to the secondary stage and supplementation of fetal bovine serum, FSH or activin to the culture medium were not effective (Gigli *et al.* 2006).

2.3.2 Tissue Grafting

The revascularization of a tissue graft is one of the most crucial steps in the functional restoration and to assess the health of the tissue. One of the main obstacles in

successful transplantation of tissues is ischemia (Yang *et al.* 2008) in which the restriction in blood supply to the tissues consequently lead to a shortage in oxygen and nutrients required for cell metabolism. The decreased follicular density in the grafted ovary is more related to ischemic factors rather than to cryoinjury and greater fibrosis observed in frozen-thawed tissues did not seem to affect the population or the morphology of primordial and primary follicles (Nisolle *et al.* 2000).

Although ovarian tissue contains abundant angiogenic genes and factors, the neovascularization and capillary infiltration in tissues is a time consuming phenomenon, which means that ovarian grafts may subject to hypoxia. In rodents, for example, ovarian tissue slices become revascularized within 2-3 days post transplantation (Dissen *et al.* 1994). It is estimated that denser tissue such as that of humans and large mammals would take longer time to vascularize and are thus more susceptible to ischemic damage (Kim 2006).

2.3.3 Autograft

Autotransplantation of ovarian tissue seems to be a practical and clinically acceptable method of fertility restoration in humans (Kim 2006). Successful fertility restoration in an ovariectomized sheep after autografting frozen-thawed cortical strips of its own ovarian tissue was reported (Gosden *et al.* 1994a). In humans, the first autographed frozen-banked tissue was performed in 2000 (Oktay and Karlikaya 2000). Ultrasonographic studies showed the formation of follicles in response to gonadotropin stimulation, confirming the long-term survival of the graft.

2.3.4 Xenograft

Xenotransplantation has proven to be an effective method in growing and developing immature oocytes stored in ovarian tissue. Follicle maturation was first documented following xenografting when fresh ovarian cortex from cats and ewes were grafted under the kidney capsule of SCID mice (Gosden *et al.* 1994b). The follicles that had developed over the 9-month grafting period were derived from primordial follicles surviving the graft. Success has been achieved as live young were produced from xenografted mouse ovaries (Snow *et al.* 2002). This achievement suggested oocytes grown in xenografted ovarian tissue from other species including domestic and wildlife may also be viable. While this is a useful and fascinating method of follicle maturation, major ethical issues would need to be resolved before clinical application could be suggested (Aubard 2003). The risk of zoonotic disease transmission and potential genome alteration in gametes are questions that need to be answered.

2.3.5 Grafting Site

Orthotopic transplantation is the only method available for fertility restoration and potential natural pregnancy. Pieces of ovarian cortical tissue are removed, preserved and then retransplanted to the peritoneum of the ovarian fossa or onto the ovary (Donnez *et al.* 2006). So far, only one spontaneous pregnancy in humans has been reported after orthotopic transplantation of cryopreserved ovarian tissue (Donnez *et al.* 2004).

Orthotopic transplantation of ovaries has also been reported in the chicken (Song and Silversides 2006). The cryopreservation of female avian genetics is only feasible by preserving the ovaries as oocytes and embryos are not available in egg-laying species. Young female White Leghorn chicks receiving donor ovary transplants from Barred Plymouth Rock chicks were able to produce donor-derived offspring, suggesting that the transplantation of ovarian tissue of chickens is possible if performed just after hatch (Song and Silversides 2007a).

Heterotopic transplantation has also become a site of interest in in vivo follicle development. The ovarian tissue is grafted to a more superficial site (such as under the skin) to monitor the growth and to allow for less invasive retrieval (Rodriguez-Wallberg and Oktay 2012). Developed antral follicles can then be aspirated, matured and fertilized in vitro and the embryo can be transferred. Successful oocyte retrieval has been reported in humans (Oktay *et al.* 2004) but no live births after IVF have been reported. Interestingly however, there has been a case of four pregnancies and three live births over a 5-year period after a heterotopic transplantation on the lower abdominal wall of a previously menopausal woman (Oktay *et al.* 2011). It has been suggested that perhaps the paracrine-endocrine signals from the transplanted ovary induce oocyte generation from resident stem cells in the other ovary in situ, or there is germ cell transfer between the two ovaries via the blood stream (Oktay *et al.* 2011). Heterotopic ovarian transplantation has also been useful in serial examination of ovarian structures in rabbits using ultrasound bio-microscopy (Cervantes *et al.* 2013).

2.3.6 Tissue Grafting on the Chorioallantoic Membrane (CAM)

The chorioallantoic membrane culture system is useful for the study of short-term ovarian tissue transplantation. This model is advantageous over the mice and culture methods because it avoids animal testing, costs less and the grafting procedure is much simpler (Martinez-Madrid *et al.* 2009). Many authors have reported success using the CAM system (Cushman *et al.* 2002; Gigli *et al.* 2005; Martinez-Madrid *et al.* 2009; Isachenko *et al.* 2012). It has been demonstrated that oocytes retained the ability to be activated in vitro after grafting onto the CAM, thus providing evidence that the CAM culture system may be a sufficient alternative to traditional culture systems.

The chorioallatoic membrane is the outermost extraembryonic layer lining the non-cellular shell membrane (Valdes *et al.* 2002). It consists of the ectoderm (interface with air and shell), the stroma (which contains the blood vessels) and the endoderm (interface with the allantois) (fig 2.1). The CAM is formed by the 4th or 5th day of incubation when the outer mesodermal layer of the allantois fuses with the mesodermal lining of the chorion and the network of blood vessels develops between the two layers (Deryugina and Quigley 2008). By the twelfth day of incubation, the CAM covers the entire surface of the inner shell membrane. The purpose of the CAM, from a developmental standpoint is to support the capillaries, collect excretory waste and actively transport ions (Valdes *et al.* 2002).

Fetal bovine cortical tissues beneath the CAM were grafted to assess follicular activation “in ovo” as an alternative to in vitro culture. Tissues were incubated on the CAM for 10 days and while the tissues vascularized rapidly and remained healthy, follicle activation did not occur (Cushman *et al.* 2002). This is due to the inhibitory effects of anti-müllerian hormone (AMH) secreted by the gonads of male and female chicken embryos. In

a different study, the activation of follicles in the ovaries of AMH receptor 2 knockout and wild type mice were compared following grafting under the chicken CAM. They found that activation of follicles in the AMH receptor 2 knockout ovaries were not inhibited, whereas follicle activation in the wild type was suppressed, further providing evidence that the presence of AMH does not permit follicular activation (Gigli *et al.* 2005). Despite these results, the chicken chorioallantoic membrane may still provide an alternative method of testing the post thaw viability of cryopreserved tissues, providing evidence of angiogenesis, which is not possible in the in vitro culture system. The objective of this system is not to provide a culture system prior to re-implantation of ovarian tissue, restoring fertility, but to provide a fast and relatively simple assessment of the post-thaw health of ovarian tissue. It is the only model that provides on-demand analysis of angiogenesis and grafting onto the developing CAM of the chicken embryo does not raise any legal or ethical questions, nor does it violate animal protection laws.

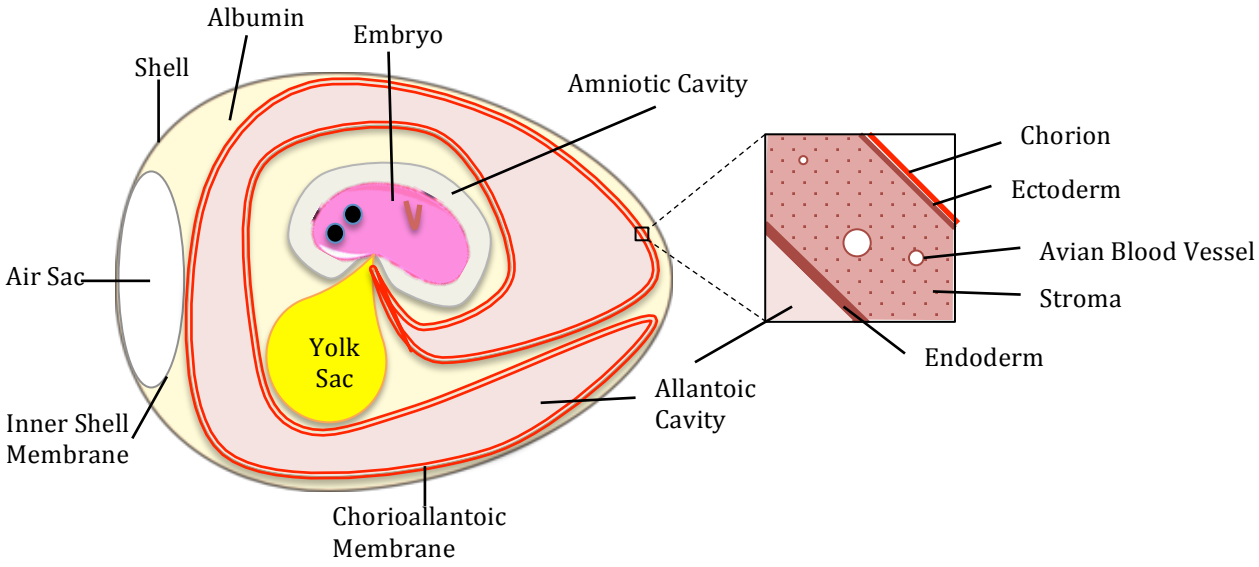

Fig 2.1. Fertilized chicken egg and cross section of CAM.

2.4 CONCLUSIONS

The loss of animal genetic resources on an international scale is occurring at an alarming rate and it is a global responsibility to ensure the safeguarding of these at-risk species.

Cryopreservation of ovarian tissue has great potential for agricultural purposes and human health. It is a useful option for the treatment of infertility and for the regeneration of rare breeds of livestock. Although success has been achieved, its efficiency and practicability are still uncertain. The challenge is to optimize the suitable protocols that can protect oocyte and ovarian structure during freezing as well as allowing oocytes to develop fully under in vivo or in vitro conditions. The goals of this thesis are to establish an effective vitrification protocol, as well as a suitable culture system to assess the post-thaw viability of bovine ovarian tissue.

2.5 HYPOTHESES AND OBJECTIVES

Study 1:

- ***Hypothesis 1***: The chorioallantoic membrane (CAM) of the chicken embryo is a more suitable culture system than traditional in vitro culture.

- ***Objective 1***: To compare the viability of bovine ovarian tissue in ovo (after grafting onto the CAM of the chicken embryo) and in vitro culture.

Study 2:

- ***Hypothesis 1***: During vitrification the higher cooling rate on the cryotop (open vitrification method) will yield better post-thaw viability of bovine ovarian tissue as compared to the cryovial (closed vitrification method)

- ***Hypothesis 2:*** Trehalose is a superior non-permeating cryoprotectant to sucrose for vitrification of bovine ovarian tissue

- ***Objective 1***: To evaluate the cooling rate of bovine ovarian tissue subjected to open and closed system devices for vitrification

- ***Objective 2***: To develop a suitable vitrification protocol for bovine ovarian tissue

CHAPTER 3: THE AVIAN CHORIOALLANTOIC MEMBRANE (CAM) CULTURE SYSTEM: A SUITABLE SHORT-TERM GRAFTING METHOD FOR BOVINE OVARIAN TISSUE

3.1 Abstract

The purpose of this study was to compare the viability of bovine ovarian tissue grafted on the chorioallantoic membrane (CAM) of chicken embryos to the traditional in vitro culture system. Bovine ovaries were retrieved from a local abattoir and cortical pieces (1-2mm^3) were randomly assigned to one of the following groups; control (fixed immediately without culture), CAM or in vitro culture. Ovarian tissues from treatment groups were removed on D1, D3 and D5 of culture, fixed, serially sectioned (5µm) and stained with H&E. The numbers of primordial, primary and secondary follicles (healthy and degenerated) per mm^3 of ovarian tissue (follicle density), and the number of infiltrated bovine and avian blood vessels were determined using standard stereological procedures. All grafts placed on the traumatized CAM demonstrated increased neovascularization over time. The density of healthy primordial follicles decreased (P =0.04) over time (13±5.2 and 27±7.1 on D1 and 7±2.3 and 11±1.4 on D5, in vitro and CAM groups, respectively) concomitant with an increase in degenerated primordial and activated preantral (primary + secondary) follicles. No difference (P =0.14) was detected between culture systems for healthy activated preantral follicle density. In CAM group, blood vessel density increased over time (p = 0.015) with increasing number of blood vessels containing avian erythrocytes. In conclusion, our results documented that the CAM of chicken embryos supported the bovine ovarian tissue grafts for 5 days and this system can complement the traditional in vitro culture (e.g. study of vascular interactions) and xenograft (e.g. simpler

to immune-compromised murine model) systems to understand the processes involved in follicle recruitment and ovarian cryopreservation.

Key Words: Ovary, in vitro culture, in ovo culture, follicle, angiogenesis, xenograft.

3.2 Introduction

From an experimental point of view, the ability to observe cells and tissues in isolation has had an incredible impact on our understanding of biological processes. When animal cells or tissues are isolated, they have to be provided with a system that closely imitates their "natural" surroundings (Willmer 2013). In vitro culture provides a controlled environment for cells and tissues to grow and develop in the presence of essential nutrients and energy sources necessary for tissue survival. In this regard, growth of follicles from primordial to ovulatory stage has been achieved in mice using an in vitro culture system (O'Brien *et al.* 2003). However, the physical and anatomical differences (size, density, etc.) in the ovaries and follicle of humans and livestock species as compared to mice would make it difficult to apply a similar culture protocol (Eppig and O'Brien 1996). While the in vitro incubation of cortical slices in large volumes of culture medium is the classical method, other procedures have been proposed to maintain excised ovarian tissues, such as xenotransplantation to an immunodeficient animal (nude or SCID mouse etc.) and grafting on the embryonic chorioallantoic membrane (CAM) within a hen's egg (Isachenko *et al.* 2012).

The CAM system is an intermediate culture system between in vitro and more complex in vivo models (including xenotransplantation). It is an economical system and does not require the use of costly hormones to prevent tissue rejection. Further, the CAM model circumvents the legal, ethical and animal welfare issues associated with use of mammalian models. The grafting procedure on the CAM is simple as compared to other xenografting. The immune system in the early chick embryo is not fully developed and therefore the risk of graft rejection is low. Compared with the in vitro system, where culture conditions are best suited for only one cell type, many different cell types can develop simultaneously on the CAM, suggesting its use for regenerative studies (Baiguera *et al.* 2012).

During fertility restoration procedure in cancer patients, the post-implantation ovarian tissue must be able to regenerate blood supply. Ovarian tissue has pro-angiogenic properties that facilitate the movement of endothelial cells to make capillaries (Rone *et al.* 1993) that are vital for follicle survival in the grafted tissue. The CAM is a multilayered structure lined by the ectodermal epithelium at the air interface, mesoderm (or stroma) containing the blood vessels and the endoderm at the interface with the allantoic sac (Valdes *et al.* 2002). The chick membranes form a bursa-like structure around the vascularized ovarian tissue creating a microenvironment similar to the one during in vivo situation (Cushman *et al.* 2002). In addition, CAM has been used to study the angiogenic properties of solid tumors (Bérubé *et al.* 2005). The extracellular matrix of the CAM is similar to the peritoneum in mammals, the most common site of orthotopic autotransplantation of ovarian tissue (Martinez-Madrid *et al.* 2009). It is therefore

reasonable to postulate that this model system can further our understanding of the physiological events involved in follicular loss by ischemia.

Another advantage of the CAM is that it is one of the well-understood avian tissues. Tissue grafting on the CAM has been used to study the development of a variety of tissues and structures for decades (Rudnick 1944; Rawles 1952) such as retinal stimulation (Leng *et al.* 2004), tissue response to biomaterials (Valdes *et al.* 2002; Borges *et al.* 2004; Azzarello *et al.* 2007) glioblastoma cells (Warnock *et al.* 2013), oncolytic adenovirus (Durupt *et al.* 2012), photodynamic therapy (Nowak-Sliwinska *et al.* 2010) and endometriosis grafts (Juhasz-Böss *et al.* 2010). Recently, follicular development has been studied using CAM culture of ovarian tissue in various mammalian species such as human (Isachenko *et al.* 2012 ; Martinez-Madrid *et al.* 2009), mouse (Gigli *et al.* 2005), and cattle (Cushman *et al.* 2002; Gigli *et al.* 2005).

The main objectives of this study were to assess the capability of the CAM to support bovine ovarian tissue through angiogenesis and to compare two culture systems, the CAM culture system and the conventional in vitro culture system for their ability to maintain follicular health and development.

3.3 Materials and Methods

3.3.1 Treatment groups and tissue collection

Bovine ovaries were collected from a local abbatoir and brought to the laboratory within 6 hours. Ovaries were stripped of surrounding fat and fibrous tissue, and washed

twice in 0.9% sterile saline. Small ovaries (n=4) without a corpus luteum were selected for dissection and the cortex was removed with a No. 22 scalpel blade. Experiment was replicated 4 times within 2-month period (i.e., one ovary on each of the 4 replication days). The cortical tissue was removed and cut into 1-2mm^2 x 0.5-1 mm thick fragments in Dulbecco's phosphate buffer saline (DPBS). Tissue fragments were then randomly assigned to: control (untreated fresh tissue without culture), in vitro culture and CAM groups, as explained below.

From each ovary 3 tissue fragments were destined for control, 9 for in vitro culture, and 9 for CAM culture. Two additional fragments from each ovary were plunged into liquid nitrogen without exposure to cryoprotectants and thawed in 1X DPBS (freezing-thawing cycle repeated 3 times for each fragment) and assigned to the CAM group as a negative angiogenesis control. One tissue fragment was grafted per egg (n=11 per replicate).

3.3.2 Control group

From each ovary 3 tissue fragments were destined for the control group. Tissues fragments were dissected from the ovarian cortex and placed immediately in 4% paraformaldehyde.

3.3.3 In vitro culture

Ovarian tissue fragments (n=9) were placed on tissue culture inserts (0.4μm pore size) in 6-well plates (Catalog numbers 353090 and 353502, respectively; Corning, USA) containing 1.2mL of culture media composed of TCM199 supplemented with 1% insulin +

transferrin + selenium (ITS; Gibco, UK), 100 mIU/ml recombinant follicle stimulating hormone (rFSH or Gonal-f; Serono, Switzerland), 100 µg/mL penicillin, 100 µg/mL streptomycin (Vetec, Rio de Janeiro, Brazil). The culture was performed at 37°C with 5% CO_2 in a humidified incubator. Tissues were removed from culture on Day 1, 3 and 5 and fixed for histological analysis as explained below.

3.3.4 CAM model and grafting procedure

The chorioallantoic membrane of the chicken embryo was exposed and processed following the procedure described earlier (Martinez-Madrid *et al.* 2009) with some modifications. Fertilized Leghorn eggs were procured from the University of Saskatchewan Poultry Centre and brought to the laboratory. The eggs were gently wiped with 70% (v/v) ethanol and incubated at 37°C and 62% relative humidity in a commercial egg incubator (1502 sportsman cabinet incubator / hatcher combo; GQF Manufacturing Co.) with the shelves moving, allowing the eggs to turn. Three days after the incubation, the eggs were candled to locate the embryonic disc, and then a window (approximately 1 x 2 cm) was made in the eggshell by surgical scissors. Albumin (2mL) was removed by aspiration with an 18-gauge needle and 5mL syringe directed toward the apex of the egg to avoid harming the developing embryo. A piece of clear tape was placed over the window to prevent dehydration, and the eggs were placed back in the incubator with the window facing upward and the shelves in a stationary position. Embryos were checked every 48h to assess their level of development and live/dead status determined by heartbeat, CAM vessel integrity and movement of the embryo.

On 10th day of incubation (defined as Day 0 of grafting), a small area of the CAM was gently traumatized by quickly touching a sterilized piece of lens paper (38.5 mm^2) dipped in sterile acetone on the CAM. This procedure is expected to remove the top epithelial layer exposing the underlying blood vessels while keeping the basal layer intact. Fragment of the bovine ovarian cortex was gently placed on the traumatized area (in ovo graft) using sterile microsurgical forceps. Tissues were removed and the embryos were sacrificed on Day 1, 3 and 5 post-grafting for histological analysis.

3.3.5 Histology of ovarian tissue

For histological investigation, the control and cultured pieces of ovarian tissue were fixed in 4% paraformaldehyde, embedded in paraffin wax, serially sectioned at 5µm, stained with hematoxylin/eosin and analyzed under a light microscope. From each tissue block, 8 serial sections were placed on each glass slide and 8 such slides were prepared by discarding 10 intervening sections (a total of 50µm between adjacent slides). Slides were graded blindly (i.e., without the knowledge of treatment group or time point) to avoid bias. The number of healthy and degenerated follicles were classified by developmental stage (i.e. primordial, primary, secondary; please see below for definitions) and counted. To avoid recounting, a follicle was counted only once in the section where the nucleolus of the oocyte was visible. The first section on each slide acted as a reference section and was not counted; thus a total of 180µm thickness of ovarian fragment (7 sections of 5µm thickness per slide x 8 slides) spanning majority of ovarian fragment (8 slides of 35µm counting thickness + 7 inter-slide gaps of 55µm = 665µm) was counted. This counting scheme

allowed us to obtain representative follicle and vascular densities for each ovary using assumption-independent stereological method. Each ovarian section was imaged at a final magnification of 4x using a 4x Objective and area of the section and follicle numbers were determined using Fiji/Image J software (described in statistical analysis section).

Morphological classification of follicles was based on the definitions previously described (Paynter *et al.* 1999). Three types of follicles were evaluated: 1) primordial follicles: an oocyte enclosed by a single layer of flat follicular cells, 2) primary follicles: an oocyte encircled by a single layer of spherical (or cuboidal) granulosa cells and 3) secondary follicles, similar to primary follicles but the oocyte is surrounded by two or more granulosa cell layers. For the purpose of this paper, antral follicles were not evaluated because they degenerated after culture. All morphologically classified primordial follicles were considered as the resting pool and primary + secondary follicles were considered as the activated preantral follicles (recruited pool). Follicle quality was graded as follows. Grade 1 - a follicle that is spherical in shape, containing a spherical oocyte with an evenly distributed granulosa cell layer; a homogeneous ooplasm and slightly granulated nucleus containing condensed chromatin; Grade 2 - a follicle similar to grade 1 but the oocyte may be irregular in shape or without condensed chromatin and the granulosa cell layer may be pulled away from the oocyte and; Grade 3 - follicle contained a misshapen oocyte with or without nuclear vacuolation and the granulosa cell layer was either partly or fully disrupted and contained pyknotic nuclei. Grade 1 and 2 follicles described a morphologically healthy follicle, whereas grade 3 follicles denoted a degenerating follicle.

Blood vessels were counted and distinguished based on the presence of nucleated red blood cells (avian origin) or red blood cells without nuclei (bovine origin).

3.3.6 TUNEL assay

Apoptotic status of the follicles was analyzed from a subset of histologic slides by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to detect DNA fragmentation. In situ TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche Applied Sciences, Mannheim Germany), according to the manufacturers instructions. Slides were washed in xylene to remove paraffin and dehydrated in decreasing concentrations of ethanol. Slides were then washed in PBS and endogenous peroxidase was blocked with 3% (v/v) H_2O_2 in methanol for 10 min. Slides were washed again in PBS and incubated with 20µg/ml Proteinase K (Sigma-Aldrich, St Louis, USA) in 10 mM Tris/HCl (pH 7.4-8.0) for 20 min followed by another wash in PBS and 5 min incubation of 0.1% (v/v) Triton X–100 in 0.1% (v/v) sodium citrate on ice. After washing slides were incubated with the TUNEL reaction mixture (50 µl) for 1 hour at 37°C followed by three rinses of PBS. Recombinant DNAse (Qiagen) was used to initiate DNA strand breaks in the positive control, and negative control sections were incubated with labeling solution but without the enzyme solution. Slides were lightly dried and mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, USA) then analyzed under fluorescent microscope with 40x objective lens (Axioskop 40, Zeiss). Follicles with TUNEL positive nucleus were considered degenerated.

3.3.7 Statistical analysis

Area and volume of ovarian tissues used was estimated by standard stereological analysis using FIJI / ImageJ software v1.49. To determine the area of each ovarian section, the "grid" plug-in of FIJI software was used. An overlay grid of evenly spaced crosses (at 100 µm distance from each other in x and y direction) was placed randomly on the image and the crosses overlapping the image were counted. Each cross represented 10000µm^2 of area and the total number of squares was multiplied by the thickness of the section (5 µm) to determine the examined tissue volume per section. The sum of the volume of all counted sections determined the total tissue volume examined for each ovary.

Follicles were classified (primordial, primary or secondary; Grade 1, 2 or 3) and counted only in the section where the nucleolus of the oocyte was visible. Antral follicles were not evaluated. Follicle and blood vessel density was calculated by dividing the number of follicles or blood vessels detected in all counting sections by the total volume of each respective tissue fragment. Following follicle densities were recorded: all preantral (primordial+primary+secondary; all grades), healthy preantral (primordial+primary+secondary; Grade 1 and 2) and degenerated preantral (primordial+primary+secondary; Grade 3); total primordial (primordial; all grades), healthy primordial (primordial; Grade 1 and 2) and degenerated primordial (primordial; Grade 3); total activated preantral (primary+secondary; all grades), healthy activated preantral (primary+secondary; Grades 1 and 2) and degenerated activated preantral (primary+secondary; Grade 3). Blood vessel densities were recorded for vessels with avian RBC (nucleated erythrocytes detected), vessels with bovine RBC (non-nucleated erythrocytes) and all vessels (nucleated, non-nucleated or no erythrocytes) Proportion of

healthy primordial and healthy activated preantral follicles were calculated by dividing the respective healthy densities by the total follicle density for each ovarian fragment.

Densities and proportions were evaluated by ANOVA and 2x4 (culture type by culture time) repeated measures factorial design using SAS ® Enterprise Guide 4.2. Normality of the residuals was evaluated before final analysis and if the values did not meet the criterion, data were transformed using log transformation. Endpoints were summarized by mean and standard error for each group. The level of statistical significance was set at a $P < 0.05$. Replicate number (ID; 1-4), time in culture (0, 1, 3 or 5 days), culture type (1= in vitro, 2=CAM) and follicle densities were tabulated for each ovarian tissue group. Syntax of SAS program included: Proc mixed covtest; class ID day culture density; model density=time culture time*culture / DDFM=kr htype=3; repeated culture (day) /subject=ID type=??; lsmeans time / pdiff adjust=tukey; lsmeans culture / pdiff adjust=tukey; lsmeans time*culture / pdiff adjust=tukey; run. Eleven covariate matrices (variance components, compound symmetry, heterogenous compound symmetry, toeplitz, banded-toeplitz, huynh-feldt, autoregressive (1), heterogenous autoregressive (1), ante-dependence, unstructured and banded-unstructured (1)) were initially tested (by replacing the "??" in the above syntax with the covariate code) to select the optimal model type based on smallest AICC value from the mixed procedure program. If the main effects or interaction term had a P-value of ≤ 0.05, for the selected model, post-hoc comparisons were done using Tukey's adjustment.

Refer to appendices (Table B) for experimental design.

3.4 Results

3.4.1 Embryo survival

In CAM group, 121 eggs were used. The survival rate of the embryos from D3-D10 (windowing to grafting) was 89.3% (109/121). From grafting to the time of ovarian tissue retrieval (D10 to D11, D13 or D15) the embryo survival rate was 99.1% (108/109).

3.4.2 Histology of ovarian tissue

Follicle densities (number per mm^3; primordial+primary+secondary combined) of control, CAM grafted and in vitro cultured ovarian tissues (mean±SE), on Day 1, Day 3 and Day 5 are presented in Table 3.1. The density of all follicles (primordial, primary and secondary; Grade 1, 2 and 3) was greater in the CAM group than the in vitro culture group on average over the culture period ($p = 0.007$), but did not differ on any given culture day ($p > 0.095$) in either treatment group. Likewise, healthy follicle density was higher on average in CAM than the in vitro group ($p = 0.016$) and did not differ on any given culture day ($p > 0.208$) in either treatment group. The degenerated follicle density exhibited a time effect. The control group did not differ from D1 in vitro or on the CAM ($p>0.731$) however a lower number of degenerated follicles was observed after Day 3 and Day 5 of culture ($p < 0.001$). No statistical difference was evident between culture types ($p = 0.296$). All antral follicles were degenerated after culture and were not counted.

In order to assess the pattern of change during the culture time, total, total healthy and total degenerated primordial and activated preantral (primary and secondary) follicle

Table 3.1. Total, healthy and degenerated follicle densities (primordial + primary + secondary follicles combined; number of follicles per mm^3) over 5-day incubation period in the control, in vitro and CAM culture systems.

Density	Control	In Vitro			CAM				
	D0	D1	D3	D5	**AVG**	D1	D3	D5	**AVG**
Total Follicle	**100±29.5**	66±32.0	90±41.8^{A}	68±16.5	**75±17.0**	80±25.3	136±36.0^{B}	100±20.0	**105±16.2***
Healthy Follicle	**93±28.2**	54±26.6	72±35.6	47±13.4	**58±14.4**	74±25.7	110±36.3	66±17.8	**83±15.6***
Degenerated Follicle	**6±1.9^{a}**	12±6.0^{a}	17±7.2^{b}	12±5.0^{b}	**17±3.4**	6±1.5^{a}	26±2.6^{b}	34±2.8^{b}	**22±3.7**

Data presented as mean±SE

Small letters (a,b) denote difference among days within a culture system.

Capital letters (A,B) represent difference between in vitro and CAM on a given culture day.

Asterisks (*) indicate difference between in vitro and CAM on average (AVG) over the entire culture period.

Statistical significance was set at $p < 0.05$

densities as well as the proportion of healthy primordial and activated preantral follicles were compared by 2 x 4 factorial design (considering control group data as Day 0 for both treatment groups) and results are presented in Fig 3.1. The total ($p<0.001$) and healthy ($p=0.041$) primordial follicle density decreased over time. Degenerated primordial ($P=0.041$) density increased by Day 3 with almost 2-fold decrease in proportion of healthy primordial follicles recorded by Day 5 ($P<0.001$; Fig. 3.1G). Overall, total, healthy and degenerated primordial follicle density in the CAM group was higher ($p=0.004$, $p <0.001$, $p=0.036$, respectively) than the in vitro group.

Unlike the total primordial follicle density, where a clear decrease was observed over time, the total activated preantral follicle density remained relatively stable over the culture period. The number of healthy activated preantral follicles had a variable pattern ($p=0.016$) of change over time (Day 3 higher than Day 1 and 5; $P<0.05$). The number of degenerated activated preantral follicles increased ($p<0.001$) over the 5-day culture period, but the proportion of healthy activated preantral follicles did not change over time. For activated preantral follicles, the culture system had no effect on the total, healthy and degenerated follicle densities or the proportion of healthy activated preantral follicles.

3.4.3 Angiogenesis

Angiogenesis (as shown by small vessels moving toward the graft in a pinwheel-like fashion) was evident at Day 3 of culture and well established by Day 5 (vessels increased in size and number around the graft over time) (Fig 3.3). The histological examination of ovarian

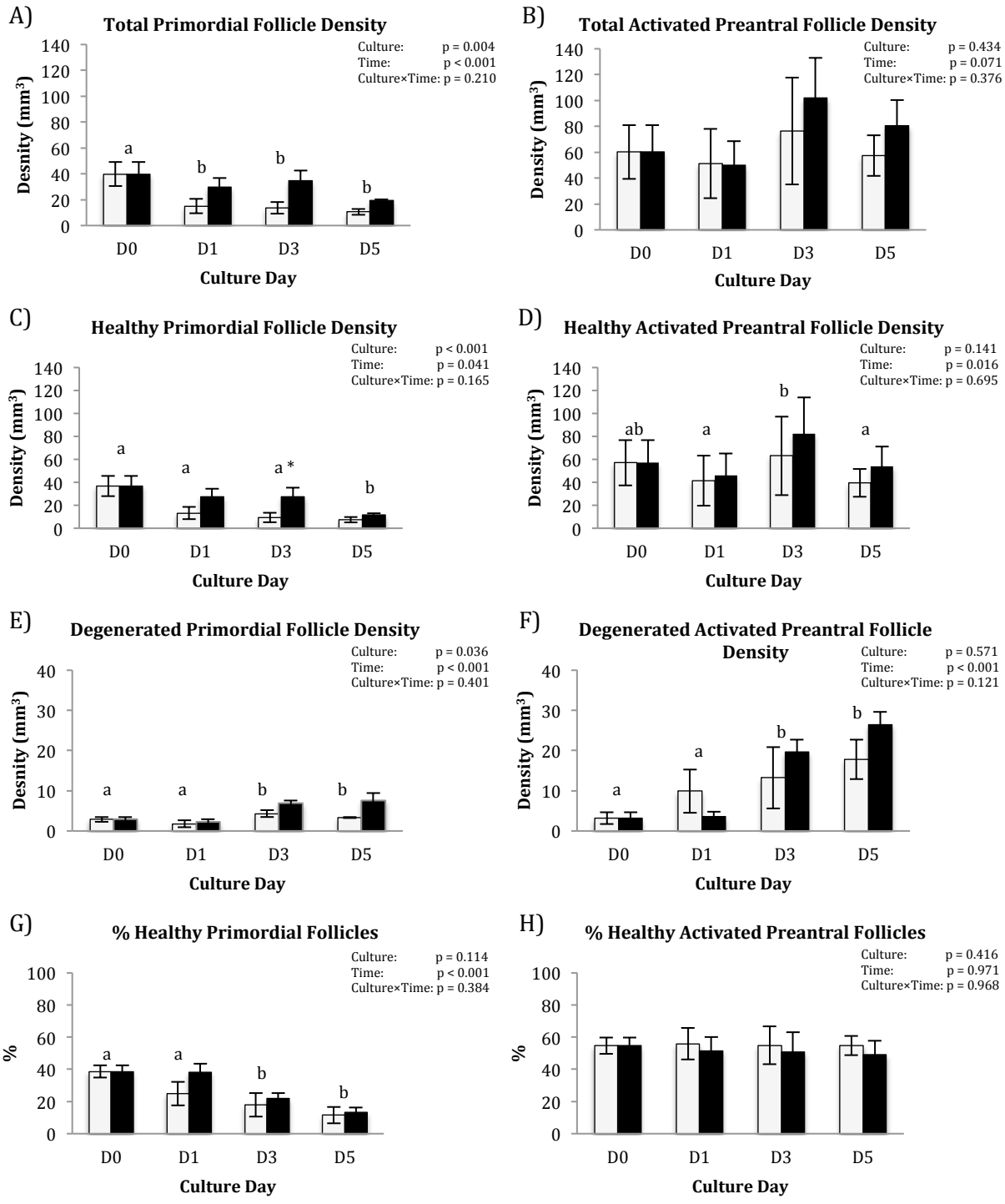

Fig 3.1. Total (A-B), healthy (C-D) and degenerated (E-F) primordial and activated preantral (primary and secondary) follicle densities and percentage of healthy primordial (G) and activated preantral (H) follicles of the total number of follicles over 5-day incubation period in the in vitro and CAM culture systems.
Each bar represents mean±SE; White bars represent in vitro culture and black bars represent CAM grafting. Letters (a,b) denote difference among culture days (D0-D5) and asterisk (*) denotes difference between in vitro and CAM systems. Statistical significance set at $p < 0.05$. Numerical means±SE are presented in appendices (Table C)

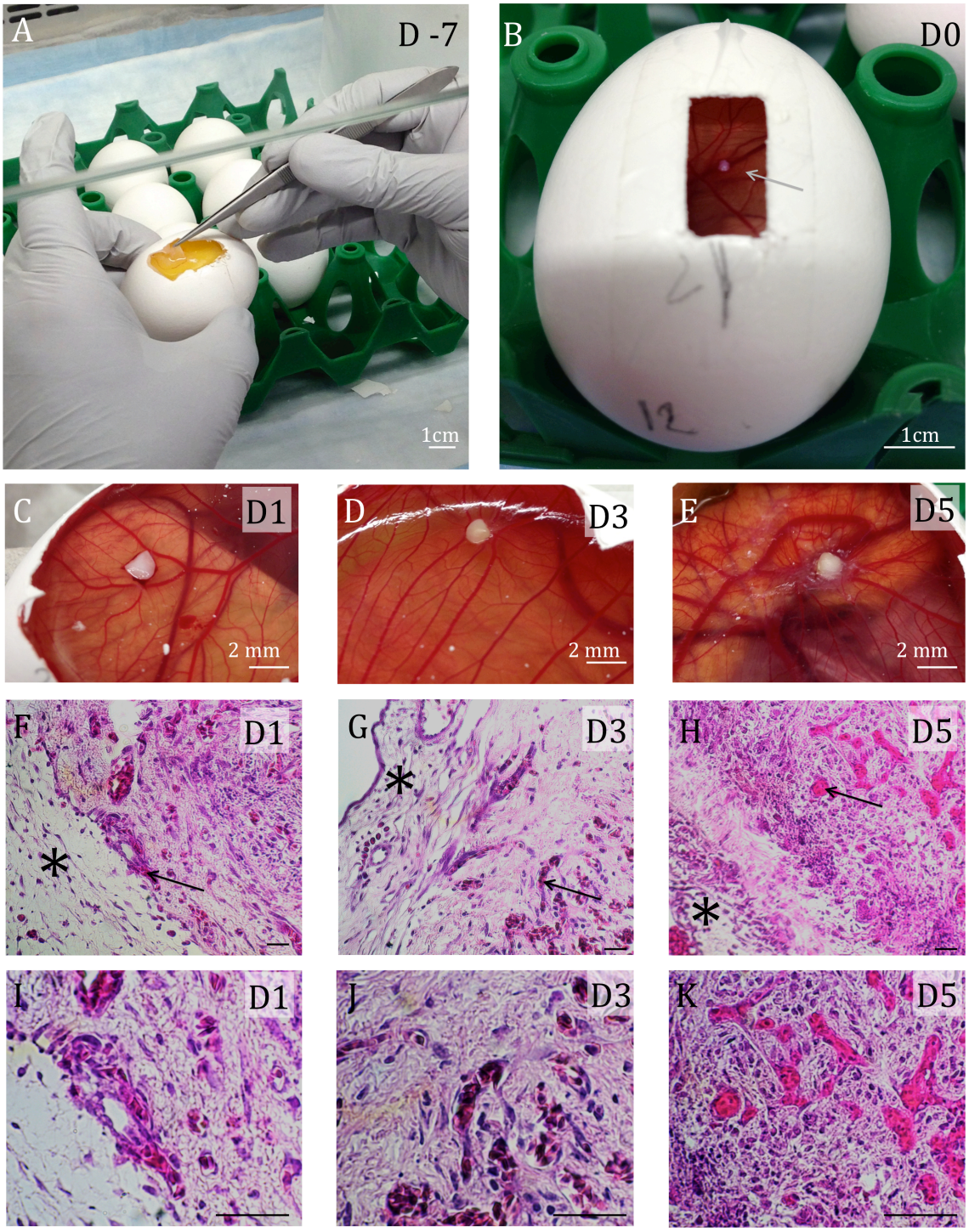

Fig 3.2. Egg processing, tissue grafting and angiogenesis. A 1x2 cm (approximately) window was made in the eggshell on D3 of incubation (D-7 of grafting) (A) and a 1-2mm^3 fragment of ovarian tissue (grey arrow) was gently placed on the traumatized CAM on D0 of experiment (B). Blood vessels developed over the grafting period in a "pinwheel-like" formation near the grafted tissue (C, D, E). Little vessel movement was observed at D1 (C, F, I) however infiltration was first observed by D3 (D, G, J), and was well established by D5 (E, H, K).
Black scale bar in histology photographs represents 50µm. Number in upper right corner of each figure indicates day of tissue grafting. Black arrows indicate avian blood vessels

grafts revealed that the number of blood vessels containing nucleated erythrocytes of chicken origin (Fig 3.2) infiltrated the tissue and the density of avian blood vessels within the ovarian grafts increased over time ($p<0.001$; Fig 3.3a) with a more than 6-fold increase between Day 3 and 5. There was a decrease over time in the density of blood vessels containing erythrocytes of bovine origin ($p<0.001$; Fig 3.3b) in both the CAM (4.05±0.29, 3.08±0.44 and 2.75±0.24 for Days 1, 3 and 5) and the in vitro group (2.57±0.26, 1.85±0.26 and 1.13 ±0.14, respectively).

In tissues damaged by liquid nitrogen, negligible number of CAM blood vessels moved toward the ovarian grafts in a pinwheel-like manner but no vessel infiltration occurred over 5 days of culture.

3.4.4 TUNEL assay

A TUNEL assay was used to confirm the follicle grading system as healthy or degenerated. Fluorescent-labeled pyknotic cells, an indication of follicular degeneration, were visible (Fig 3.4).

3.5 Discussion

The objective of this study was to compare the follicular growth and survival of fresh bovine ovarian tissue following in vitro vs. CAM short-term culture as well as neovascularization of bovine ovarian tissue on the CAM. In the present study, preantral

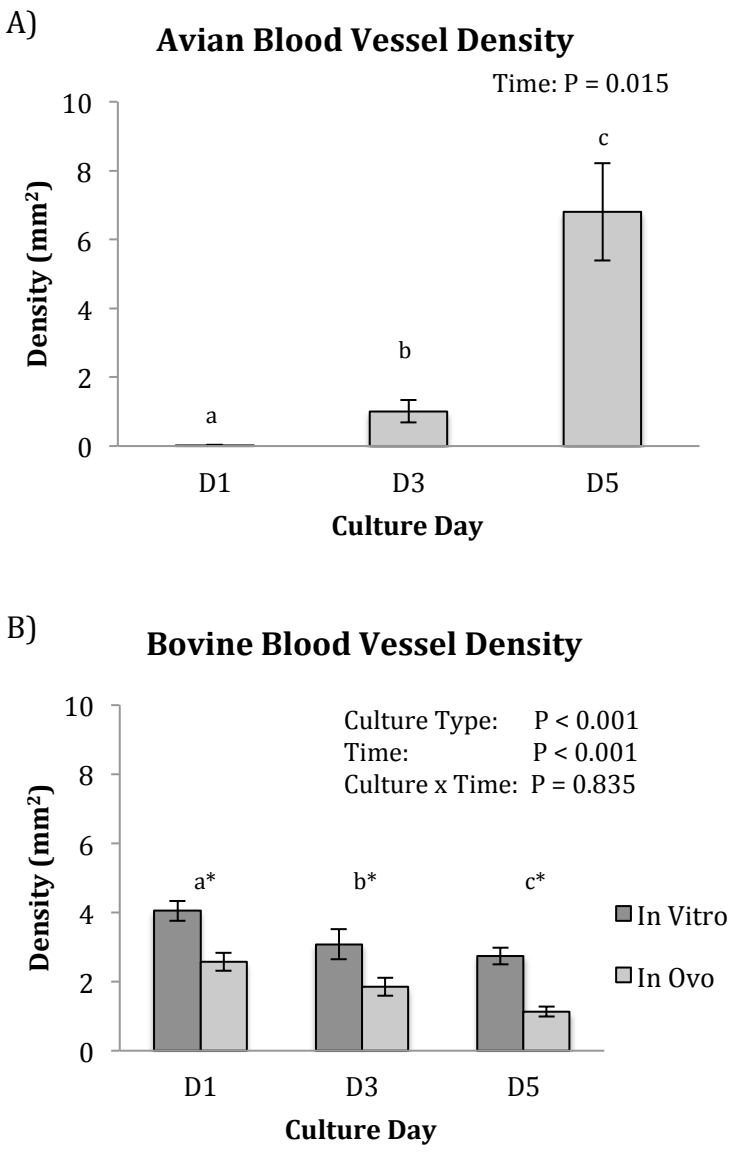

Fig 3.3. Density of blood vessels containing nucleated red blood cells, indicating avian origin (A) and blood vessels containing enucleated red blood cells, indicating bovine origin (B). Letters (a,b,c) denote difference among culture days (D1, D3, D5) and asterisk (*) denotes difference between in vitro and in ovo (CAM) on a given day.
Statistical significance was set at $p<0.05$.

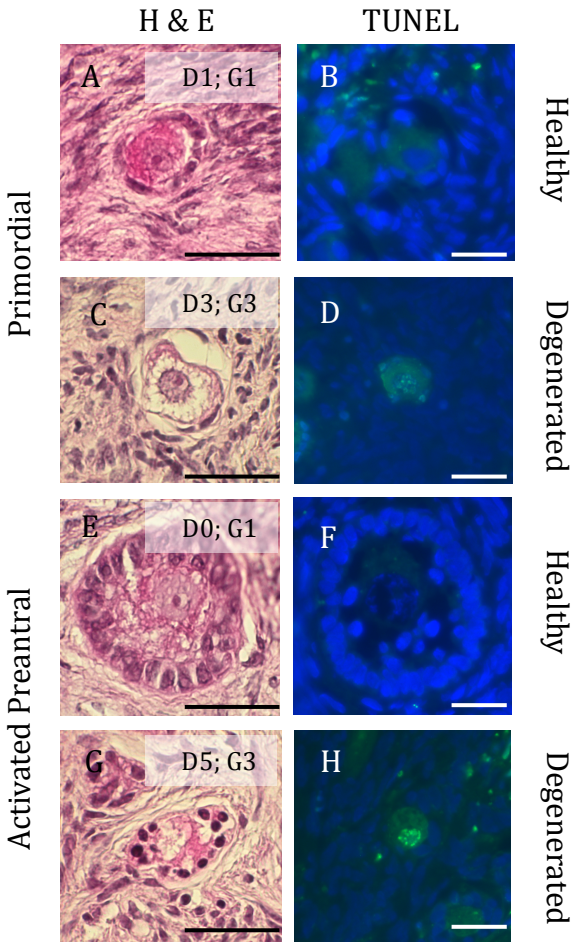

Fig 3.4. Histological micrograph of healthy (A, B, E, F) and degenerated (C, D, G, H) primordial (A, B, C, D) and activated preantral (E, F, G, H) follicles. Follicles were immunostained with TUNEL (B, D, F, H) and then stained with H&E (A, C, E, G). Green fluorescence indicated a positive TUNEL test. Blue indicated cells stained with DAPI. Scale bar represents 25μm. Number in upper right corner indicates day of grating and grade of follicle.

follicles were healthy in both the in vitro and CAM groups. In general, the follicle density in the CAM group tended to be higher than the in vitro group. Total primordial follicle density tended to decrease over time while the total activated preantral follicle density tended to increase. Culture time had an effect in all groups where healthy follicle density decreased over the culture period and degenerated follicle density increased. Likewise, the proportion of healthy primordial follicles decreased over time, whereas the proportion of healthy activated preantral follicles was not affected by time. Angiogenesis was evident in all tissues grafted to the CAM 3 days post grafting and blood vessels were well established in the graft after 5 days. Blood vessels containing nucleated erythrocytes (indicating avian origin) increased over the culture period while the blood vessel density of vessels containing bovine erythrocytes decreased over time.

Chicken embryo survival rate was very high throughout this study, indicating that CAM model is tolerant to and accepting of manipulation. As other vertebrates, chickens are protected by a dual immune system comprised of T cells, controlling antibodies, and B cells, controlling cell-mediated immunity. T cells can first be detected by Day 11 of incubation and B cells by Day 12. After Day 15 of incubation, the B cell population begins to diversify and by Day 18, the embryo becomes immunocompetent (Janse and Jeurissen 1991). Other studies report a similar survival rate of tissues placed onto the traumatized CAM (Martinez-Madrid *et al.* 2009; Isachenko *et al.* 2012).

This study confirmed that healthy follicles were present in both culture groups. In vitro culture is a traditional method of assessing the effect of any treatment on cell or tissue health. The damages to cells or tissues due to any treatment are manifested over the incubation time. The extra-embryonic blood vessels of the chorioallantoic membrane are

not innervated, similar to the placenta in mammals, thus it provides a suitable xenotransplantation site for a variety of tissues. For its cost-effectiveness, ease of use and the success with other tissue types, we hypothesized that culture on the chorioallantoic membrane of a chicken embryo would be a suitable alternative to traditional culture systems. Our results showed that the morphological quality of the follicles was independent of the culture conditions, thus the CAM system was as suitable as the in vitro system. This is in agreement with other findings using human ovaries (Isachenko *et al.* 2012), and fetal bovine ovaries (Cushman *et al.* 2002).

The total and healthy follicle densities were highest on Day 3 of incubation. Although the difference was not statistically significant among culture days, we expected that the healthy follicle density would decrease over the culture period. Although tissue samples were randomly distributed the variability among replicates observed in the study may be attributed to the biological difference among individuals. It is anticipated that ovaries collected from an abattoir belong to cows differing in age, stage of their estrous cycle and from different farms with diverse feeding and management regimens. In humans, predicting ovarian follicle densities based on biopsies are extremely varied due to uneven distribution of follicles over the surface of the ovary (Lambalk *et al.* 2004). The random distribution of ovarian fragments should ensure that fragments of high and low follicle density are evenly dispersed among culture types and days of culture. The higher follicle density observed on Day 3 of incubation can be attributed to random allocation of higher follicle density fragments.

The ovaries used in this study were from mature cows. We observed that the proportion of primordial follicles was lower than that of activated preantral follicles. This

finding is in agreement with another study in humans in which the follicle dynamics were age-dependent and there was a negative correlation between primordial follicle density and age. The total number of follicles also decreases with age; causing a shift in the preantral follicle pool from mostly primordial to more primary and secondary follicle dominated (Faddy and Gosden 1995).

We compared the total number of each primordial and activated preantral follicles as well as the number of healthy and degenerated follicles. Our results indicated that the healthy primordial follicle density decreased over time in both culture groups while the degenerated primordial follicle density increased. One of the main problems following transplantation of ovarian tissue is the massive loss of follicles during the ischemic period until revascularization can be established (Rahimi *et al.* 2010). In larger tissue fragments, follicle degeneration can also be attributed to hypoxia (Hartshorne 1997). Culture of fresh ovarian tissue can thus be said to have detrimental effects on preantral follicles (Faustino *et al.* 2014). The CAM provides an experimental system in which primary follicles can be maintained in a quiescent state in a readily accessible environment. The number of primordial follicles in fetal bovine ovaries decreased concomitant with an increase in primary follicles in a 7-day culture system, indicating follicle activation, however there was no difference in the number of activated preantral follicles over the culture period and the spontaneous activation observed was inhibited in CAM (Fortune *et al.* 2000). In our study, we did not observe any difference in the activated preantral follicle density between the CAM and the in vitro system. A decrease in the primordial follicle population over time was however detected in both culture systems, with a greater decrease in the in vitro culture

system. This difference could be attributed to the origin of the ovaries (fetal vs. adult ovaries used in our study) and the status of the chorioallantoic membrane upon grafting.

The total number of primordial follicles and their proportion relative to the total number of follicles decreased over time. Like the primordial follicles, the healthy follicle density had a tendency to decrease over time while the number of degenerated activated preantral follicles increased. As a whole, neither the total follicle density nor the proportion of activated preantral follicles of the total changed significantly over the culture period in both culture systems.

The decrease in primordial follicle density with concurrent stability of activated preantral follicle density indicates a potential activation of primordial follicles occurring in the ovarian tissue. This finding differs from other reports of ovarian tissue culture on the CAM as it has been shown that the presence of circulating anti-Müllerian hormone (AMH), which is naturally occurring in the chick embryo, inhibits follicle activation in fetal bovine and newborn murine ovarian tissue (Cushman *et al.* 2002; Gigli *et al.* 2005). Other studies have shown similar results where the addition of AMH to in vitro culture inhibited the transition of primordial follicles to primary follicles (Durlinger *et al.* 1999; Carlsson *et al.* 2006) and the actions of stimulatory factors (such as kit-ligand and fibroblast growth factor) (Nilsson *et al.* 2007). In contrast, AMH enhanced FSH-stimulated growth of rat preantral follicles in vitro (McGee *et al.* 2001). These differences may be explained by a species to species variation or even age (fetal and newborn vs. adult), which needs to be explored further.

Vascularization of the ovarian tissues grafted to the CAM was well established by Day 5. We observed a dramatic increase in the number of avian blood vessels containing nucleated blood cells between tissue collection Day 3 and Day 5. This is in agreement with other studies in which the CAM was used as an alternative culture system. Earlier, chicken blood vessels infiltrated in bovine (Cushman *et al.* 2002) and human (Martinez-Madrid *et al.* 2009; Isachenko *et al.* 2012) ovarian tissue over the culture period.

Chorioallantoic membrane culture provides angiogenesis as an additional indication of tissue viability. As shown in our study, ovarian tissues damaged by liquid nitrogen did not vascularize to the same extent as fresh undamaged tissues. Vascularization is very important to avoid ischemia in tissues during incubation. Blood vessels supply the tissues with the nutrients, oxygen and regulatory signals required for tissue survival. This is particularly important for primordial and early activated preantral follicles in the ovarian cortex as they do not possess an independent vasculature at that stage and must therefore rely on nearby blood vessels (Delgado-Rosas *et al.* 2009). More recently it has been shown that increasing the depth of ovarian tissue grafts to include ovarian medullar tissue leads to better vascularization and is crucial for neo-angiogenesis (Isachenko *et al.* 2012).

The development of a reliable cryopreservation protocol for ovarian tissue is important for assisted reproduction in both human fertility clinics and for the conservation of female animal genetic resources (Lucci *et al.* 2004). Proper cryopreservation requires adequate penetration of cryoprotectants with minimum toxicity and optimal cooling and thawing rates. While progress has been made in minimizing cryoinjury, cryopreservation involves a large number of variables and thus, no single approach is guaranteed to be universally effective and accepted (Shaw *et al.* 2000). The damages induced by

cryopreservation become increasingly more evident over time (Keros *et al.* 2007), thus the usage of the chorioallantoic membrane culture system will aid in the development of a more universal cryopreservation protocol for bovine ovarian tissue by providing a simple and more economical post-warming culture method.

In conclusion, the CAM supports bovine ovarian tissue. Healthy follicles were present in both the in vitro and CAM groups showing that the CAM can be used as an alternate biological system to culture bovine ovarian tissue. Secondly, angiogenesis may be an additional indicator of tissue health. All fresh tissues grafted to the CAM underwent neo-angiogenesis and were infiltrated with blood vessels (increase in blood vessel density over the culture period) while tissues damaged by liquid nitrogen did not undergo angiogenesis.

CHAPTER 4: THE EFFECTS OF CRYOPROTECTANTS AND COOLING DEVICES ON THE SURVIVAL OF VITRIFIED BOVINE OVARIAN TISSUE GRAFTED TO THE AVIAN CHORIOALLANTOIC MEMBRANE

4.1 Abstract

The purpose of this study was to assess the viability of bovine ovarian tissue vitrified using two non-permeating cryoprotectants (sucrose and trehalose) and two cryodevices (cryotop and cryovial). The ovarian cortical tissue (n=5 ovaries) was cut into 1-2mm^3 fragments and assigned to one of 7 treatment groups. Non-vitrified (control) or vitrified tissue fragments were grafted on the chorioallantoic membrane of 10-day old chick embryos for 5 days to examine the changes in number of healthy and degenerated preantral follicles. Tissues were vitrified in TCM199 supplemented with 15% ethylene glycol, 15% DMSO, 20% calf serum and 0.5M sucrose or trehalose then placed in a cryovial or on a cryotop. After warming, the vitrified tissues were either immediately placed in 10% formalin (control) or grafted. Follicles from control and vitrified tissue were observed under a light microscope for healthy morphology and the densities (number/mm^3) of healthy and degenerated preantral (primordial, primary and secondary) follicles were determined by standard stereological procedures. Healthy, degenerated or total (healthy+degenerated) follicle densities did not differ ($p>0.08$) between cryoprotectants or cryodevices. Proportion of healthy follicles was higher ($p<0.001$) in the control than in treatment groups. All grafts placed on the traumatized CAM demonstrated neovascularization to the CAM after 5 days, but no difference was observed for blood vessel density among treatments. In conclusion, sucrose and trehalose, and the cryotop and cryovial were equally effective in vitrifying bovine ovarian tissue.

Key Words: CAM, cattle, chick embryo, cryodamage, cryotop, cryovial, follicle, ovary, sucrose, trehalose, vitrification, , xenograft

4.2 Introduction

Developing a reliable method for maintaining the viability of immature oocytes in ovarian tissue for future growth and development is important for the conservation of female genetics. From the standpoint of conservation of animal genetic resources, the maintenance of ovarian tissue maximizes the female genetic potential of endangered and valuable animals by virtue of possessing its rich population of immature follicles.

Unlike fully-grown oocytes, the largest mammalian cell, immature follicles are more suitable for cryopreservation because oocytes are small in size, the zona pellucida is absent and they are undifferentiated and metabolically quiescent (Newton *et al.* 1996). Furthermore, because of the long growing phase, the oocytes have more time to repair sub-lethal damages to organelles (Shaw *et al.* 2000).

Cryopreservation involves an almost infinite number of variables and thus there is currently no universal protocol for ovarian tissue cryopreservation. Vitrification, where cells are exposed to high concentrations of cryoprotectants and frozen at an ultra-rapid cooling rate, transforms the viscous portion of the cell to a glass like state, avoiding harmful intracellular ice formation. Due to its simplicity and success rate, this method has become more popular in recent years.

During vitrification, cells are exposed to high concentrations of permeating cryoprotectants, which can be toxic as these molecules are small enough to enter the cells

and interact with the cell organelles. (Fahy *et al.* 1990), The use of non-permeating cryoprotectants in vitrification media allows for an increase in the total concentration of cryoprotectants without drastically increasing the potential toxicity of the solution. These disaccharides help to dehydrate the cells/tissues by increasing the extracellular osmolarity (Posillico *et al.* 2010). Follicle preservation depends on the nature and concentration of the cryoprotectants used as well as the rate at which they are removed during the thawing process to avoid damage incurred by osmotic swelling during warming/thawing (Neto *et al.* 2008).

Vitrification uses ultra-rapid cooling and warming rates to pass through the dangerous temperature zone (between 15°C and -5°C) more quickly, decreasing chilling injury and ultimately decreasing potential toxic and osmotic damage (Massip 2003; Orief *et al.* 2005) Several cryodevices have been designed to achieve a rapid cooling rate in ovarian tissue such as the cryovial, plastic straw, cryotube, electron microscope and copper grids, cryostraw, cryobag, and the specially designed ovarian tissue cryosystem (OTC).

Vitrification systems can be broadly classified into two distinct categories according to specimen exposure to liquid nitrogen: open systems (direct contact of tissue with LN_2) and closed systems (no direct contact with LN_2). Open systems (such as the cryotop) offer an increased cooling rate, however those systems might present a risk of contamination and transmission of disease (Cutting *et al.* 2009). Closed systems (such as the cryovial) allow a more sterile vitrification, but at the expense of a decreased cooling rate.

Traditionally, in vitro culture and xenografting have been used to study ovarian tissue development and post-thaw tissue integrity. Tissue incubation on the chorioallantoic membrane (CAM) within a hens egg offers an alternative method of short-term culture. Our

preliminary work indicates that follicles within fresh ovarian tissue grafted to the CAM are healthy after 5 days of culture.

The objective of this study was to distinguish the effects of two non-permeating cryoprotectants (sucrose and trehalose) as well as two cryodevices (cryotop and cryovial) on the morphology of preantral follicles and tissue neovascularization after cryopreservation and 5-day CAM culture.

4.3 Materials and Methods

4.3.1 Treatment groups and tissue collection

Bovine ovaries (n=5) from a local abattoir were collected and brought to the lab within 6 hours. The surrounding fat and connective tissue was removed and ovaries were washed twice in 1X Dulbecco's phosphate buffer saline (DPBS). Small ovaries (n=5) without a corpus luteum were chosen for dissection and the cortex was removed with a scalpel blade (No. 22). Cortical pieces were further cut into 1-2mm^2 x 0.5-1 mm thick pieces in 1X DPBS. Pieces were them randomly assigned to control group or vitrified group.

From each ovary, 42 tissue pieces were distributed as follows: 18 for control and 24 for vitrification. Table 4.1 organizes tissue fragments into their respective groups. Control tissues were either kept in DPBS or treated with vitrification media containing sucrose or trehalose but were not vitrified (3 groups; n=6 pieces each). In the vitrified groups, tissues were treated with vitrification media containing either sucrose or trehalose and then vitrified using either the cryotop or the cryovial (4 groups; n=6 pieces each).

Table 4.1: Treatment groups for bovine ovarian tissue fragments

Treatment Group	CAM Culture (yes/no)	Non-Permeating Cryoprotectant used	Device (Cryotop / Cryovial)	Number of tissue fragments (1-2mm^3)
1. Control-Control	No (D0)	None	None	3
2. CP Control-Sucrose	No (D0)	Sucrose	None	3
3. CP Control-Trehalose	No (D0)	Trehalose	None	3
4. Vit-Control-S-Cryotop	No (D0)	Sucrose	Cryotop	3
5. Vit-Control-T-Cryotop	No (D0)	Trehalose	Cryotop	3
6. Vit-Control-S-Cryovial	No (D0)	Sucrose	Cryovial	3
7. Vit-Control-T-Cryovial	No (D0)	Trehalose	Cryovial	3
8. Culture-Control	Yes (D5)	None	None	3
9. CP Culture-Sucrose	Yes (D5)	Sucrose	None	3
10. CP Culture-Trehalose	Yes (D5)	Trehalose	None	3
11. Vit- Culture -S-Cryotop	Yes (D5)	Sucrose	Cryotop	3
12. Vit- Culture -T-Cryotop	Yes (D5)	Trehalose	Cryotop	3
13. Vit- Culture -S-Cryovial	Yes (D5)	Sucrose	Cryovial	3
14. Vit- Culture -T-Cryovial	Yes (D5)	Trehalose	Cryovial	3

CP: Cryoprotectant; D: Day; S: Sucrose; T: Trehalose; Vit: Vitrified

4.3.2 Vitrification and thawing of bovine ovarian tissue

Tissue pieces were placed (at room temperature) in 5mL of the vitrification (equilibration) solution 1 (VS1) containing TCM199 supplemented with ethylene glycol (EG; 7.5% v/v), dimethyl sulfoxide (DMSO; 7.5% v/v), and calf serum (CS; 20% v/v) for 10 minutes, followed by 5 min in 5mL of the vitrification solution 2 (VS2) containing TCM199 with EG (15% v/v), DMSO (15% v/v), calf serum (20% v/v) and 0.5M of either sucrose or trehalose. Tissues with minimal surrounding VS2 solution were placed along the inner wall

of a 1.5mL cryogenic vial (cryovial: Nalge Nunc International, Rochester NY, USA) and sealed or placed on a cryotop (Kitazato Supply Co., Fujinomiya, Japan). Both the cryovial and the cryotop were held over liquid nitrogen vapour for 1 second and then plunged into liquid nitrogen (LN_2) and stored for 1h.

For warming, cryovials were removed for the LN_2, held at room temperature for 10 seconds, cap was removed and vial was held in a water bath at 37°C. Warming solution 1 (TCM199 media containing calf serum (20% v/v) and 0.5M sucrose or trehalose; 1ml; 37°C) was immediately added to the vial. Tissues were then removed after 2 min and washed twice in 2 mL of plain warming solution (TCM199 and 20% calf serum, no cryoprotectants). Cryotops were removed from the LN_2, held in air for 10 seconds, and then placed in 1mL of warming solution 1 for 2 minutes. As with the cryovial, tissues were removed and washed twice in 2 mL plain warming solution. Tissues were kept in the last washing solution until the time of grafting or fixing in 4% formalin (within 10 minutes).

4.3.3 Thermocouple analysis of cooling and thawing

The cooling rates of tissues on the cryotop and within the cryovial were assessed using a 1.3 mm Digi-Sense Type K, thermocouple wire (Kapton insulated probe; Cole Palmer, Montréal, Canada) connected to a 1529 Chub-E4 Thermometer Readout machine (Fluke – Hart Scientific, American Fork, Utah, USA). A device developed by Kleinhans *et al.* (2010) provided a model for a simple cryotop freezing method using a glass microscope slide. Measurements were recorded every 0.5 seconds and then downloaded to the computer using HyperTerminal ® in Windows XP software as per the user manual guidelines of the read-out machine.

4.3.4 CAM model and grafting procedure

The chorioallantoic membrane was processed following the procedure previously published by Martinez-Madrid *et al.* (2009) with some modifications. Fertilized Cornish-Cross eggs were obtained from Lilydale Inc. (Wynyard, SK, Canada) via a local hatchery, brought to the lab, lightly wiped with 70% ethanol and placed in a commercial egg incubator (1500 series, GQF Manufacturing Company, Savannah GA, USA) at 37°C and 62% relative humidity with the shelves moving, allowing the eggs to turn. On Day-3 of incubation, the eggs were candled to find the embryonic disk, and then a rectangular window (approximately 1 x 2 cm) was made in the eggshell by surgical scissors. Two milliliters of albumin was aspirated with an 18-gauge needle and 5mL syringe through the window from the apex part of the egg, to avoid the developing embryo. A piece of tape was placed over the window to prevent dehydration, and the eggs were placed back in the incubator with the window facing upward and the shelves in a stationary position. These eggs were checked every 48h to assess their development and live / dead status determined by heartbeat, CAM vessel integrity and movement of the embryo.

On Day 10, a small area of the CAM was gently traumatized by quickly touching a sterilized circular disc of lens paper (38.5mm^2) dipped in sterile acetone on the CAM to remove the top epithelial layer and to expose the underlying blood vessels. A 1-2mm^3 piece of bovine ovarian cortex was carefully and gently placed on the traumatized area using microsurgical forceps. Tissues were removed after 5 days for histological analysis and the embryos were sacrificed.

4.3.5 Histology of ovarian tissue

For histological investigation, the control and cultured pieces of ovarian tissue were fixed in 4% paraformaldehyde, embedded in paraffin wax, serially sectioned at 5µm, stained with hematoxylin / eosin and analyzed under a light microscope. From each tissue block, 8 serial sections were placed on each glass slide and 4 such slides were prepared by discarding 10 intervening sections (a total of 50µm between adjacent slides). Slides were graded blindly (i.e., without the knowledge of treatment group) to avoid bias. The number of healthy and degenerated follicles were classified by developmental stage (i.e. primordial, primary, secondary; please see below for definitions) and counted. To avoid recounting, a follicle was counted only once in the section where the nucleolus of the oocyte was visible. The first section on each slide acted as a reference section and was not counted; thus a total of 140µm thickness of ovarian fragment (7 sections of 5µm thickness per slide x 4 slides) spanning majority of ovarian fragment (4 slides of 35µm counting thickness + 3 inter-slide gaps of 55µm = 305µm) was counted. This counting scheme allowed us to obtain representative follicle and vascular densities for each ovary using assumption-independent design-based stereologic method. Follicles were counted and classified at a magnification of 40x and each ovarian section was imaged at a final magnification of 4x using a 4x Objective and area of the section and follicle numbers were determined using Fiji/Image J software (described in statistical analysis section).

Morphological classification of follicles was based on the grading protocol previously described (Paynter *et al.* 1999). Three types of follicles were evaluated: 1)

primordial follicles: an oocyte enclosed by a single layer of flat follicular cells, 2) primary follicles: an oocyte encircled by a single layer of spherical (or cuboidal) granulosa cells and 3) secondary follicles, similar to primary follicles but the oocyte is surrounded by two or more granulosa cell layers. For the purpose of this paper, antral follicles were not evaluated; morphologically classified primordial follicles were considered as the resting pool, and primary + secondary follicles were considered as the activated preantral follicles (recruited pool). Follicle quality was graded as follows. Grade 1 - a follicle that is spherical in shape, containing a spherical oocyte with an evenly distributed granulosa cell layer; a homogeneous cytoplasm and slightly granulated nucleus containing condensed chromatin; Grade 2 - a follicle similar to grade 1 but the oocyte may be irregular in shape or without condensed chromatin and the granulosa cell layer may be pulled away from the follicle and; Grade 3 - follicle contained a misshapen oocyte with or without nuclear vacuolation and the granulosa cell layer was either partly or fully disrupted and contained pyknotic nuclei. Grade 1 and 2 follicles described a healthy follicle, whereas grade 3 follicles denoted a degenerating follicle

Blood vessels were distinguished in the ovarian stroma by the presence of nucleated (indicating avian origin) or non-nucleated (bovine) erythrocytes. The numbers were recorded for vessels with avian RBC, vessels with bovine RBC and all vessels (nucleated, non-nucleated or no erythrocytes).

4.3.6 Statistical analysis

Area and volume of ovarian tissues was estimated by standard stereological analysis using FIJI / ImageJ software. To determine the volume of the ovarian tissue sections the "grid" plug-in, an analysis tool in FIJI, provided a means to overlay a grid of evenly spaced crosses onto the image. The grid was placed randomly on the image and the crosses overlapping the image were counted. Each cross represented 10000μm^2 of space and the total number of squares was multiplied by the thickness of the section (5 μm) to determine the volume. The sum of the volume of all 28 sections determined the total ovarian tissue volume.

Follicle and blood vessel density was calculated by dividing the number of follicles or blood vessels detected in all counting sections by the total volume of each respective tissue fragment. Following follicle densities were recorded: total preantral (primordial+primary+secondary; all grades), healthy preantral (primordial+primary+secondary; Grade 1 and 2) and degenerated preantral (primordial+primary+secondary; Grade 3). Blood vessel densities were recorded for vessels with avian RBC (nucleated erythrocytes detected), vessels with bovine RBC (non-nucleated erythrocytes) and all vessels (nucleated, non-nucleated or no erythrocytes). The proportion of healthy preantral follicles was calculated by dividing the healthy density by the total follicle density for each ovarian fragment. Antral follicles were not counted in the analysis as all antral follicles were degenerated after culture.

Follicle densities and proportions were evaluated by one-way repeated measures ANOVA using SAS ® Enterprise Guide 4.2. Normality of the residuals was evaluated before final analysis and if the values did not meet the criterion, data were transformed using log

transformation. The level of statistical significance was set at a $P < 0.05$. Replicate number (ID; 1-5), time in culture (0 or 5 days), CP (1= sucrose, 2=trehalose), device (1=cryotop, 2=cryovial) and follicle densities were tabulated for each ovarian tissue group. Syntax of SAS program included: Proc mixed covtest; class ID time CP density; model density= CP / DDFM=kr htype=3; repeated CP (time) /subject=ID type=??; run. Eleven covariate matrices (variance components, compound symmetry, heterogenous compound symmetry, toeplitz, banded-toeplitz, huynh-feldt, autoregressive (1), heterogenous autoregressive (1), ante-dependence, unstructured and banded-unstructured (1)) were initially tested (by replacing the "??" in the above syntax with the covariate code) to select the optimal model type based on smallest AICC value from the mixed procedure program. If the main effects or interaction term had a P-value of ≤ 0.05, for the selected model, post-hoc comparisons were done using Tukey's adjustment. Endpoints were summarized by mean and standard error within replicates.

Refer to appendices (Table C) for experimental design.

4.4 Results

The cooling rate of ovarian tissue fragments on the cryotop and in the cryovial was 7481±206° C/min and 664±26° C/min respectively (Fig 4.1).

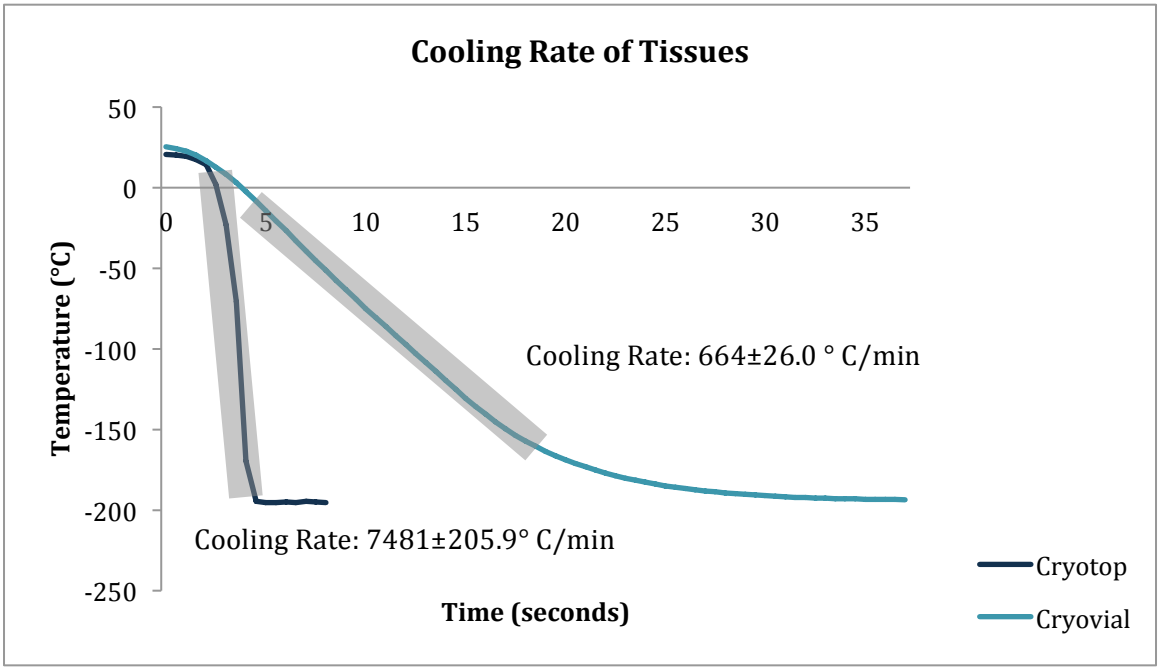

Fig 4.1. Cooling rate (mean±SE) of tissues on cryodevices (n=3 measurements per device). Measurements were taken every 0.5 seconds.
Rectangles represent the duration of measurement for each respective cryodevice.

4.4.1 Histology of ovarian tissue

Histological examination after 5-day of ovarian tissue grafting on CAM showed that primordial, primary and secondary preantral follicles were healthy. The antral follicles were degenerated by the end of grafting period and were not included in the data analysis (Fig 4.2). An initial analysis was done to examine the differences among control tissues (pre-culture, ie: those that were not placed on CAM, both vitrified and non vitrified). Since no difference ($p > 0.05$) was observed, data from these groups were combined and pooled data set was renamed as control group for further analyses.

The total, healthy and degenerated follicle densities as well as the proportion of healthy follicles in different cryodevices and cryoprotectants are presented in Fig 4.3 and Fig 4.4. No differences were detected ($P > 0.08$) between the cryovial and the cryotop for any follicle density. The proportion of healthy preantral follicles was higher in the control group as compared to the cryodevices ($p = 0.006$), indicating a culture effect (Fig 4.3).
Likewise, no difference was observed between sucrose and trehalose for any follicle parameter. The degenerated preantral follicle density in the sucrose group was higher than the control group ($p = 0.049$) but no difference was observed between the control and sucrose, trehalose or the cultured (D5) control, nor was there statistical difference among sucrose, trehalose or the cultured (D5) control. The proportion of healthy preantral follicles decreased in the cultured control, sucrose and trehalose groups as compared to the non-cultured control ($p < 0.001$) indicating a culture effect (Fig 4.4).

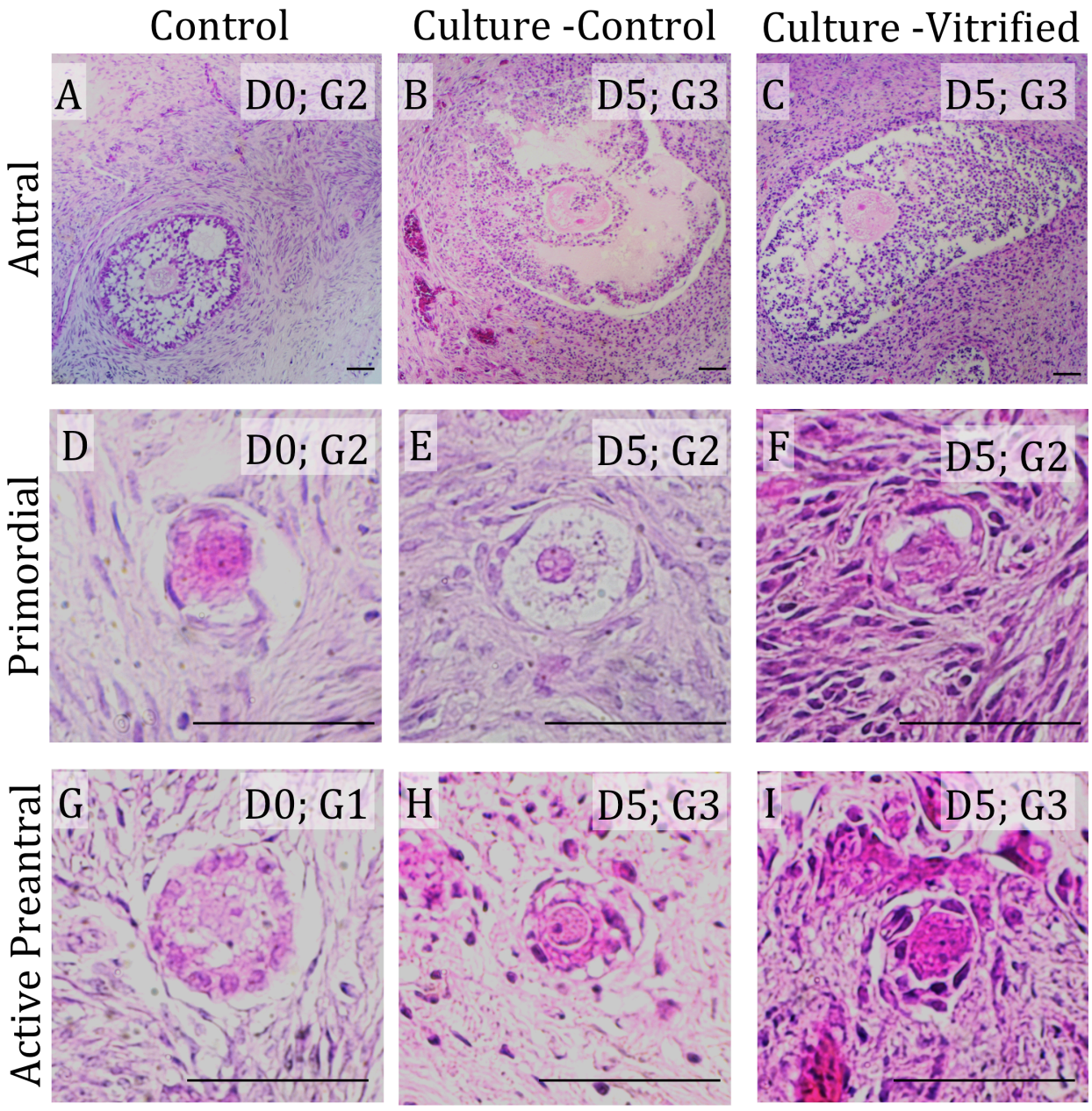

Fig 4.2. Examples of antral (A-C), primordial (D-F) and active preantral (G-I), follicles in control (A, D, G), culture-control (non-vitrified; B, E, H) and culture-vitrified (C, F, I) bovine ovarian tissue. Antral follicles were degenerated in 5-day culture (B, C) and were therefore not counted in this study.
Scale bar represents 50μm. Number in upper right corner of each figure indicates day of tissue grafting and grade of follicle.

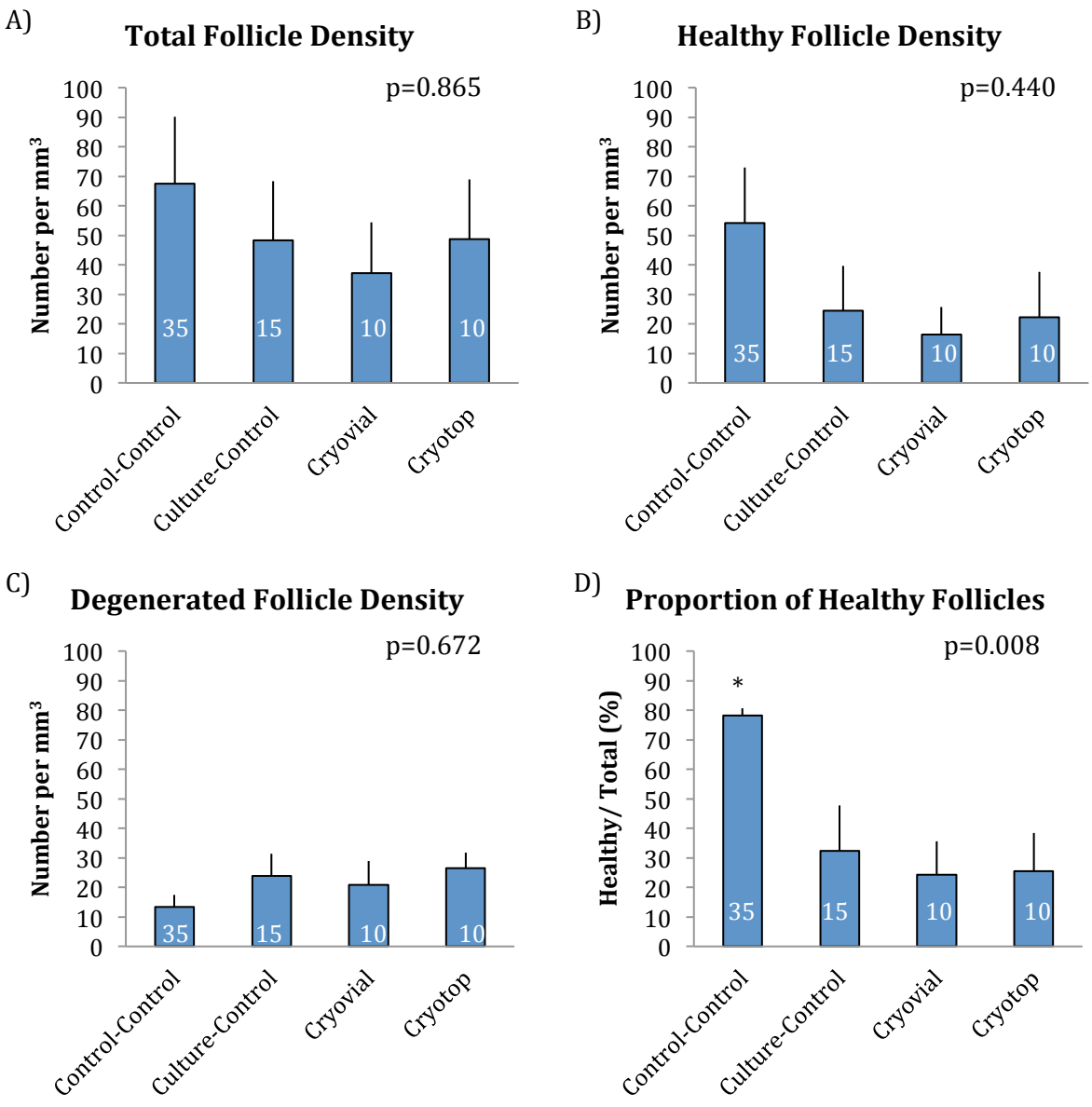

Fig 4.3. Total (A), healthy (B) and degenerated (C) follicle density (number per mm^3) and the proportion of healthy follicles (D) among controls and cryodevices after 5 days of CAM culture [1]

Each bar represents mean±SE. Asterisk (*) denotes statistical difference among groups. The numbers in bar graphs represent N value (number of treatment groups over 5 replicates). Statistical significance was set at $p<0.05$.

[1] Data presented in graphs are pooled, treatments included in each graph are (from table 4.1):

Control-Control: Treatments 1-7
Culture Control: Treatments 8-10
Cryovial: Treatments 13-14
Cryotop: Treatments 11-12

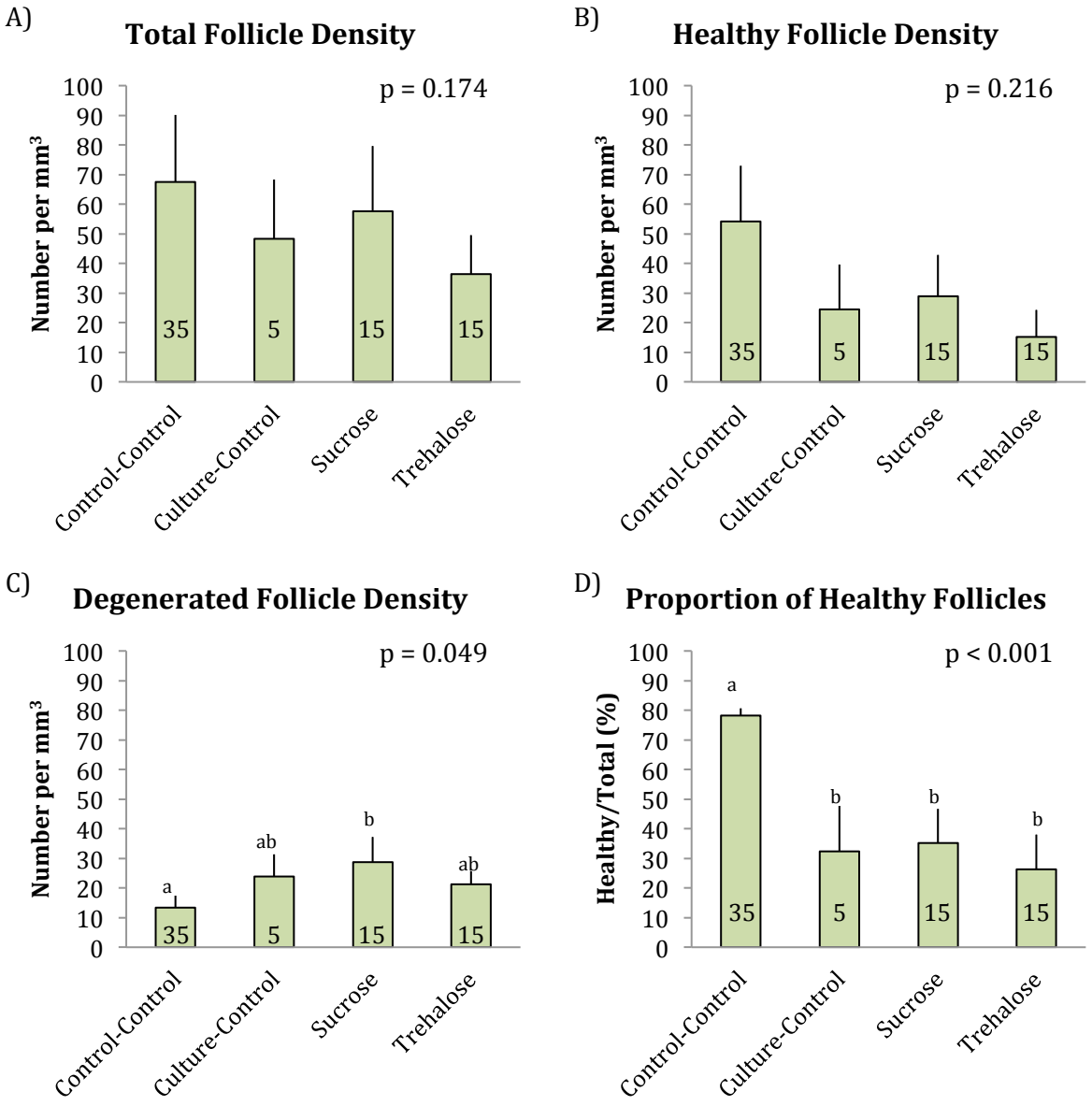

Fig 4.4. Total (A), healthy (B) and degenerated (C) follicle density and the proportion of healthy follicles (D) among controls and cryoprotectants after 5 days of CAM culture. [2] Each bar represents mean±SE. Letters (a,b) denote statistical difference among groups. The numbers in bar graphs represent N value (number of treatment groups over 5 replicates). Statistical significance was set at $p<0.05$.

[2] Data presented in graphs are pooled, treatments included in each graph are (from table 4.1):
Control-Control: Treatments 1-7
Culture Control: Treatment 8
Sucrose: Treatments 9, 11, 13
Trehalose: Treatments 10, 12, 14

4.4.2 Angiogenesis

Avian vasculature converged in a pinwheel like fashion toward the graft, and vessels were well established in the graft by day-5 (Fig 4.5). Results of blood vessel infiltration are summarized in Fig 4.5. All grafts placed on the CAM showed adhesion to the CAM over time. The control and vitrified groups did not differ. Cryoprotectants (sucrose and trehalose) were not different ($p=0.38$) nor was a difference observed between cryodevices (cryotop and cryovial) ($p=0.65$).

4.4.3 Embryo survival

The survival rate of the chick embryos was 93.9% (108/115) during the CAM culture period. There was no correlation between those that did not survive and the type of graft received over the 5-day grafting period.

4.5 Discussion

The objective of this study was to compare the effects of sucrose and trehalose, and the use of the cryotop and the cryovial, on the survival and health of preantral follicles and tissue neovascularization in the previously vitrified ovarian tissue at the end of 5-day grafting period on the chorioallantoic membrane of chick embryos. Angiogenesis was evident in all treatment groups over the 5-day CAM grafting period indicating that tissues

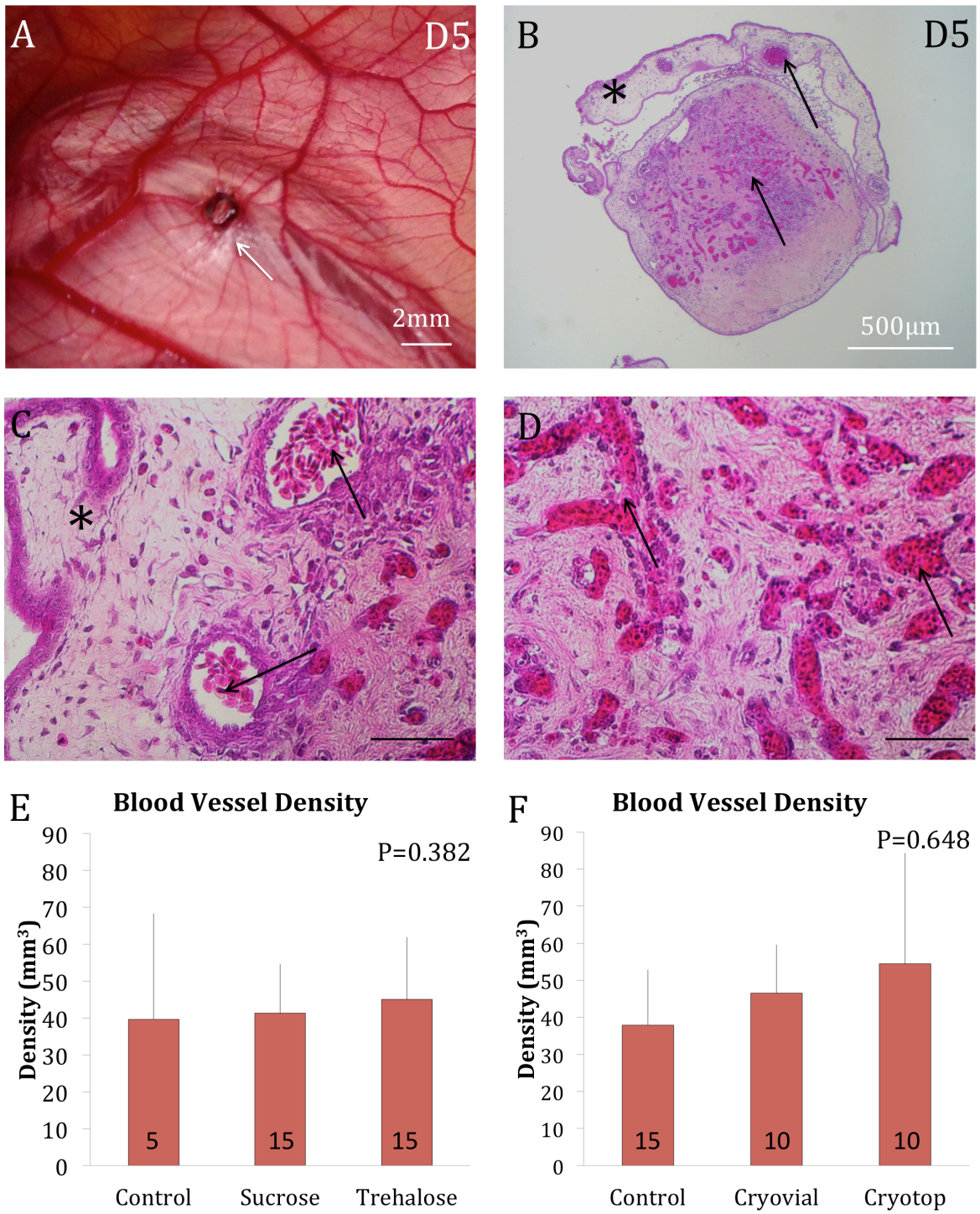

Fig 4.5. Small capillaries converging toward the tissue graft (A) supporting it 5 days post grafting to the CAM; white arrow indicates tissue graft. Histological evaluation shows that the tissue fragment is well vascularized by avian blood vessels (B) and these vessels are present in the CAM (C) and the inner core of the tissue (D). Bar graphs of avian blood vessel density in control (culture control) and cryoprotectant groups (E) and in control (culture control and CP Control) and cryodevice groups (F) grafted to the CAM for 5 days. Each bar represents mean±SE.
Black scale bar represents 50µm. Numbers in upper right corner indicate day of grafting. Asterisk (*) represents the CAM, black arrows indicate avian blood vessels.

were viable, but no statistical difference was observed in the blood vessel densities among treatment groups. In the previous study, tissues damaged by liquid nitrogen (without cryoprotectants) did not have avian vessel infiltration or "pinwheel-like" movement of vessels toward graft. In this study however, all tissues were infiltrated with avian blood vessels and a characteristic pinwheel formation was observed around all tissue grafts, indicating that these tissues were healthy.

There was no difference between cryoprotectants, nor was there a difference between cryodevices, despite the vast difference in cooling rate using these devices. We documented a more than 2-fold decline in the proportion of healthy follicles between the non-cultured control group and the CAM groups, indicating that 5-day xenografting has a detrimental effect on tissues. Vitrified and unvitrified tissues on Day 5 did not differ, indicating that the damages incurred are due to culture (Fig 4.4).

The carrier system is one of the most important factors in successful vitrification as it determines the cooling rate (Huang *et al.* 2008). It has been reported that a cooling rate of approximately 2500°C/min can achieve a vitrified state (Palasz and Mapletoft 1996) however the cooling rate varies due to sample nature and size, cryoprotectants, volume of vitrification solution surrounding the sample and cryodevice used. To our knowledge, there are very few reports on the cooling rate of vitrified ovarian tissue. A cooling rate of 17,000 °C/min has been observed on a metal cryotop (Kagawa *et al.* 2009). Our thermocouple reader accurately measured the temperature every 0.5 seconds. Kleinhans *et al.* (2010) determined a cooling rate up to 10^5 °C/min in a 0.1µl aqueous sample. The cooling rate of tissue fragments used in this study was low as they were more dense and larger in size. A high embryo survival at a cooling rate of 69000°C/min has also been reported (Seki *et al.*

2014). In the same study, only a slight reduction in embryo survival was observed at 880°C/min but dropped to 50% at 95°C/min. In our study, the decrease in the proportion of healthy follicles can be attributed to tissue culture due to a significant difference between cultured control and non-cultured control groups.

Our results indicated a non-significant difference between cryodevices for follicle morphology. We expected that cooling on the cryotop would yield a higher proportion of healthy follicles as compared to the cryovial. The cryotop is an open system, meaning the ovarian tissue fragment is in direct contact with the liquid nitrogen. While this allows for a more rapid cooling rate, there is also a risk of contamination from liquid nitrogen that is not sterile. A closed system such as the cryovial would ensure a sterile environment, however the thick plastic wall of the cryovial is a non-conductive material, thus is could decrease cooling rate and negatively affect follicle survival (Amorim *et al.* 2011).

Additives with large molecular weights such as sucrose and trehalose can significantly reduce the total amount of cryoprotectants required, as well as the toxicity for successful cryopreservation (Liebermann *et al.* 2002). Compared to other sugars, trehalose seems to have a higher capacity to preserve biomolecules and protect cells by binding more tightly to water (Crowe *et al.* 1996; Sano *et al.* 1999). In our study, no statistical difference between trehalose and sucrose for follicle morphology was observed. Contrary to our results, the morphological preservation of preantral follicles in rabbits increased in the presence of sucrose compared to trehalose (Neto *et al.* 2008). Also, while the number of viable follicles in the control sucrose group was lower than the trehalose control group, the proportion of healthy follicles was higher in sucrose. Similar results were reported for human ovarian tissue (Tian *et al.* 2015). A trend favouring trehalose in human ovarian

tissues suggests that trehalose may be a better sugar than sucrose for primordial follicle cryopreservation. In our study, the total, healthy and degenerated follicle density, as well as the proportion of healthy follicles was slightly higher (numerically) in the sucrose group, however the difference from the trehalose group did not reach statistical significant.

Angiogenesis of the tissues grafts in this study was evident in all treatment groups. The density of blood vessels containing bovine erythrocytes did not change in vitrified or non-frozen fresh ovarian tissues during the 5-day grafting period. A radial arrangement of blood vessels converging toward the tissue concurrent with an increase in vessel numbers around the graft is considered to be evidence of neoangiogenesis (Baiguera *et al.* 2012). Rapid revascularization of tissues is important to minimize follicle loss due to ischemic damages (Kagawa *et al.* 2009). The "living" ovary produces angiogenic factors, which facilitate the movement of endothelial cells in the graft (Rone *et al.* 1993). Levels of mRNA coding for angiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoetin-2 (Angpt-2) decreased significantly following the cryopreservation of mouse ovaries, however expression was higher in vitrified groups than in slow freezing (Choi *et al.* 2013). We postulated that damaged tissues would produce less angiogenic factors, that contributing to a lesser blood vessel infiltration. However, our study there was no difference in the vasculature between the vitrified and the control group, thus we can speculate that our vitrification system was successful. Vessel infiltration was well established by the end of CAM grafting (Day-5), which agrees with other short-term CAM studies of ovarian tissues (Martinez-Madrid *et al.* 2009; Isachenko *et al.* 2012)

In conclusion, the results presented in this study suggest that vitrification of bovine ovarian tissue is a promising technique for the conservation of the female germ line. No

difference was observed between sucrose and trehalose, or between the cryotop and the cryovial neither for the morphological normality of preantral follicles nor for angiogenesis of the ovarian tissue grafts. Both treatments and devices were equally as effective for maintaining preantral follicles in bovine ovarian tissue after vitrification, which did not differ from fresh tissues. Based on our present findings of equal effect between the cryotop and the cryovial, the latter device is recommended to avoid the direct contact of tissue with liquid nitrogen.

CHAPTER 5: GENERAL DISCUSSION

On a global scale, genetic diversity in farm animals is under threat. In the past century, change and selection pressures by environmental factors and human intervention have caused a loss in net diversity and an increased rate of extinction in many rare breeds of livestock. Efforts have been made to rectify the current situation and such strategies would benefit from the use of assisted reproductive techniques such as artificial insemination (AI) in vitro fertilization (IVF) and genome resource banking (Andrabi and Maxwell 2007). From a conservation point of view, the manipulation of the ovaries is particularly interesting because of the enormous supply of immature gametes, a wealth of female genetic potential (Comizzoli and Wildt 2014).

The experiments presented in this thesis were undertaken in an attempt to develop methods for the successful vitrification of bovine ovarian tissue and to apply a suitable culture system in order to assess the post warming viability of the tissue fragments. We assessed two culture systems, the chorioallantoic membrane (CAM) (in ovo) culture system and the in vitro culture system on neovascularization (in the former) and preantral follicle quality over a short-term culture period. We also examined the vitrification of bovine ovarian tissue fragments using open (cryotop) and closed (cryovial) cryodevices and media containing one of two non-permeating cryoprotectants (sucrose and trehalose).

We first explored the possibility of using a biological system to culture fragments of ovarian tissue by comparing the CAM of a hens egg to traditional in vitro culture. Our objective was to study the capability of the CAM to support bovine ovarian tissue through angiogenesis and to compare follicular heath and development in CAM and in vitro culture

systems. We hypothesized that the CAM system was a more suitable culture system for bovine ovarian tissue than the in vitro culture system. We determined that there was no statistical difference between the CAM and in vitro culture systems for the proportion of healthy primordial and activated preantral follicles. We also observed a marked increase in neovascularization of all tissues grafted onto the CAM over time. Therefore, the CAM supported bovine ovarian tissues, and the healthy follicles present in tissues cultured in both the CAM and the in vitro culture systems indicate that the CAM can be used as alternative to in vitro culture. Consequently the CAM was used in subsequent studies.

The development of a suitable culture system is essential for successful ovarian tissue as damages incurred during the cryopreservation process only become evident after culture. Culture is an effective way to assess post-warming damages. Follicles have to be able to grow and develop, and tissues need to be able to revascularize to reestablish fertility. Ovarian tissue culture is important for understanding how follicles differentiate and develop.

The CAM is advantageous to the in vitro culture system in a number of ways. It is a biological system with a rather simple grafting procedure and it is cost effective, not requiring expensive hormones (Isachenko *et al.* 2012). The objective of this system is not to provide a culture system prior to re-implantation of ovarian tissue, restoring fertility, but to provide a fast and relatively simple assessment of the post-thaw health of ovarian tissue. It is the only model that provides on-demand analysis of angiogenesis and grafting onto the developing CAM of the chicken embryo does not raise any legal or ethical questions, nor does it violate animal protection laws.

Next, we explored parameters of vitrification on bovine ovarian tissue. The successful cryopreservation of ovarian tissue has a wide variety of applications from human and veterinary medicine, to biotechnology and genetics. Although there are many challenges to overcome in the pursuit of a standardized and universal freezing protocol, each discovery unlocks new information that helps put the puzzle together.

Vitrification is a promising method to cryopreserve ovarian tissue. It is a fast an inexpensive means of cryopreservation that does not require specific equipment. Tissues are cooled at an ultra rapid rate, transforming the aqueous compartments of the cell to a solid non-crystalline solid or "glass-like", hence avoiding damaging intracellular ice crystal formation (Rall 1987).

The carrier system is one of the most important factors in vitrification as it determines the cooling rate (Huang *et al.* 2008). We hypothesized that the higher cooling rate in an open vitrification system would be more efficient and yield better post-warming viability of bovine ovarian tissue than a closed vitrification system. The cryotop has been designed to minimize the volume of vitrification solution surrounding the tissues in order to achieve ultra-rapid cooling however it poses biosecurity risks because tissues are exposed directly to potentially contaminated liquid nitrogen. Alternatively the cryovial can be used to avoid this risk but the cooling rate is not as rapid as with the cryotop due to the thickness of the vials plastic wall (Amorim *et al.* 2011). Given the results of our thermocouple experiment, we were correct in hypothesizing that the use of the cryotop would lead to higher cooling rates than the cryovial.

The addition of non-permeating cryoprotectants to a vitrification medium can significantly reduce the concentration of permeating cryoprotectants, thus reducing the

toxicity of a vitrification solution (Liebermann *et al.* 2002). Trehalose binds more tightly to water molecules (Crowe *et al.* 1996; Sano *et al.* 1999), thus we hypothesized that trehalose would be a superior non-permeating cryoprotectant to sucrose to maintain normal morphology of follicles in bovine ovarian tissue. No statistical difference was observed between sugars for follicle morphology in this study.

In this thesis, angiogenesis was evident in all fresh and cryopreserved tissues grafted to the chorioallantoic membrane. Rapid revascularization of the tissues is essential for minimizing follicle death associated with ischemia (Kagawa *et al.* 2009). Angiogenic factors in the ovary have been shown to decrease after cryopreservation in mouse ovaries (Choi *et al.* 2013), contributing to a lesser blood infiltration. We observed no statistical difference between the vessel infiltration of control and vitrified tissues, thus we can speculate that out vitrification system was successful.

Three objectives were undertaken in this thesis: First, to compare the viability of bovine ovarian tissue in ovo (after grafting onto the CAM of the chicken embryo) and in vitro culture; Second, to evaluate the cooling rate of bovine ovarian tissue subjected to open and closed system devices for vitrification; and Third: to develop a suitable vitrification protocol for bovine ovarian tissue. We hypothesized, respectively, that the chorioallantoic membrane (CAM) of the chicken embryo is a more suitable culture system than traditional in vitro culture, that during vitrification the higher cooling rate on the cryotop (open vitrification method) will yield better post-thaw viability of bovine ovarian tissue as compared to the cryovial (closed vitrification method) and finally that trehalose is a superior non-permeating cryoprotectant to sucrose for vitrification of bovine ovarian tissue. Overall, we were successful in what we set out to demonstrate. We observed that

the CAM was a suitable alternative grafting method for bovine ovarian tissue, and used this system in the subsequent experiment as a result. We evaluated the cooling rate of tissues placed on two cryodevices and compared these findings, along with the follicular morphology of tissues subjected to these devices and two different non-permeating cryoprotectants. Although neither the cryodevices, nor the cryoprotectants differed in their effect on follicular morphology (rejecting our hypotheses regarding these parameters), healthy follicles were present in all treatment groups, indicating that we were successful in our third and final objective.

CHAPTER 6: CONCLUSIONS & FUTURE DIRECTIONS

The objectives executed in this thesis have lead to the following conclusions:

- Grafting on the chicken chorioallantoic membrane provides a suitable alternative method of short term culture for bovine ovarian tissue
- While the cooling rate of bovine ovarian tissue was much higher on the cryotop than in the cryovial, these devices do not differ in their ability to preserve follicular morphology post-vitrification and 5-day culture.
- Trehalose and sucrose do not differ in their ability to preserve follicular morphology post-vitrification and 5-day culture.

Future directions of bovine ovarian tissue cryopreservation should include improving our knowledge of the mechanisms of follicular growth and applying suitable cryopreservation protocols. While numerous successes have been achieved in cryopreservation of ovarian tissue, resulting in viable tissue post-warming and follicle growth after culture in vitro, understanding the needs of cryopreserved follicles to develop into viable oocytes would be an asset in the formation of protocols that not only protect the cells from cryopreservative damages, but also ensure that they are capable of further development.

From a cryopreservation standpoint, the creation of a universal freezing protocol for a variety of tissues would be highly convenient. In some respects slow freezing is better understood than vitrification, as it has been more widely researched. Many questions have yet to be answered concerning the permeation kinetics of different cryoprotectants,

optimum cryoprotectant concentration and composition as well as the ideal equilibration time so that tissues can be completely penetrated by cryoprotectants yet be free of cytotoxic effects (Amorim *et al.* 2011). Our results indicated no difference between the cryotop and the cryovial, or between sucrose and trehalose. Further replication of our vitrification may reveal a statistical difference between these parameters. Given our results, we recommend that the cryovial be used for vitrification over the cryotop. While the cooling rate is lower, tissues are protected from direct contact with potentially contaminated liquid nitrogen.

Lastly, one of the most significant challenges to preserve primordial and preantral follicles is the ability to provoke these cells to achieve full maturation and fertilization capacity in culture (Comizzoli and Wildt 2014). While it has been shown to be a feasible technique in the mouse model, resulting in live offspring (O'Brien *et al.* 2003), limited success has been achieved in other species (Songsasen *et al.* 2012). For bovine ovarian tissue, there is much potential for the formation of competent transferable embryos. To achieve this goal, the following directions are proposed:

- Develop an efficient vitrification protocol for bovine ovarian tissue using the CAM by examining other cryodevices and cryoprotectants (at varying concentrations)
- Infiltration of avian vasculature in ovarian tissue can be validated by histopathological evaluation of sectioned tissue fragments.
- Once a protocol is established on the CAM, subject bovine ovarian tissue to long-term culture (either in vitro or xenograft) to observe follicle development.
- Antral follicles formed can then be aspirated and matured and fertilized in vitro.

From a commercial point of view, the cryopreservation of bovine ovarian tissues must be as simple as possible. Vitrification is a relatively simple and inexpensive procedure for cryopreserving ovarian tissue, however the dissection of cortical tissue is tedious and delicate. Ideally the cryopreservation of whole ovaries could rectify this issue.

The studies presented in this thesis are merely stepping stones in the pursuit of successful regeneration of cryopreserved ovarian tissues and maximized genetic potential.

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APPENDICES

Table A: Cryodevices, cryoprotectants and post-thaw viability assay used in ovarian tissue vitrification in the last 15 years.

Article	Species	Tissue Size	Cryoprotectants used	Cryodevice used	Post-warm viability assay	Major Findings
Kagabu and Umezu, 2000	Mouse, Hamster, Rat, Rabbit, Monkey	Ovaries divided into 4-8 pieces	20.5% DMSO, 15.5% acetamide, 10% PROH, 6% PEG	Cryotube	Xenograft to uterus of pseudopregnant rat Histological analysis	Vitrified grafts contained morphologically normal follicles and many large antral follicles in some grafts. Uterus is a hospitable site for ovarian tissue grafts.
Sugimoto et al, 2000	Rat	Whole ovaries	20.5% DMSO, 15.5% acetamide, 10% PROH, 6% PEG	Glass test tube	Autograft, histological analysis,	Antral and preantral follicles were observed in fresh and vitrified tissues, antral follicles were smaller in vitrified group.
Al-Aghbari and Menino Jr., 2002	Sheep	5x5x1 mm	35% EG, 5% PVP, 0.4M trehalose	SSV	In vitro maturation	Sheep oocytes in vitrified ovarian tissue exhibit similar IVM rates of vitrified and non-vitrtified oocytes
Salehnia, 2002	Mouse	Whole ovaries	30% Ficoll, 0.5M Sucrose, 10.7% acetamide, 40% EG	Cryovial	Autograft, histological analysis	Morphologically normal primordial and primary follicles after 11 days of grafting, large preantral and antral follicles were degenerated after 5 days of grafting.
Isachenko et al, 2003	Human	$0.5mm^3$	1) 40% EG, 0.35M Sucrose, 5% egg yolk 2) 40% EG, 18% Ficoll 70, 0.35M sucrose 3) 10% EG and 20% Me_2SO, then 20% EG and 20% Me_2SO.	Straw, copper grid	Steroid assay (E_2, P_4), histological analysis.	Best protocol for tissues was using CP group 1 with either straw or copper grid
Migishima et al, 2003	Mouse	Whole ovaries	1M DMSO	Cryovial	Autotransplantation, IVF, ET. Histological analysis	Normal development of follicles in vitrified grafts
Hasegawa et al 2004	Mouse	Whole ovaries	15% EG, 15% DMSO, 0.5M Sucrose	Cryotop	Histological analysis, IVG, IVM, IVF	Morphology of follicles maintained, high maturation rate to MII stage, oocytes retained capacity to be maturated and fertilized in vitro
Rahimi et al, 2004	Human	0.5 x 1 x 4mm	25% GLY, 24% EG, 1% Supercool ® X-100, egg yolk	0.25mL straws, copper EM grids	Xenotransplantation into SCID mice, Lucifer yellow SV staining,	No difference in level of tissue necrosis between slow freezing and vitrification.
Bordes et al, 2005	Sheep	Hemi ovaries	2.62M DMSO, 2.60M acetamide, 1.31M PROH, 0.0075M PEG	Cryotube	Histological analysis, hormone assay (P_4), Live birth after autotransplantation	P_4 levels increased normally in all ewes, 3 pregnancies occurred (4 lambs born, 1 died of physical abnormalities). Few primordial and antral follicles on grafted ovaries evident after histological analysis
Chen et al, 2006	Mouse	1.2 x 1.5 x 1.5 mm	1) 15% EG, 15% DMSO, 0.5M sucrose (DCV) 2) 30% Ficoll 70, 0.5M sucrose, 10.7% acetamide, 40% EG (Conventional vitrification)	Cryovial (direct cover vitrification – DCV)	Histological analysis, trypan blue assay, ultra structural analysis, allogeneic orthotopic transplantation, autologous heterotopic transplantation	Percentage of normal follicles higher in DCV than conventional vitrification and slow freezing. Follicle number higher after grafting in DCV than vitrification and slow freezing.
Courbière et al, 2006	Sheep	Whole ovaries	2.75M DMSO, 2.76M formamide, 1.97 PROH	Cryobag	Histological analysis, trypan blue assay, Ultra structural analysis	Normal primordial follicle proportion fell from 83% to 48% after vitrification. More cytoplasmic damages in vitrified ovaries, nuclear damage similar in fresh and vitrified ovaries.

Gandolfi et al, 2006	Human, Cow, Pig	1mm^3	1) 5.64M EG, 5% PVP, 0.4M Trehalose 2) 3.58M EG, 2.82M DMSO	0.5ml Plastic Straw	Histological analysis	Pig ovaries more cryoresistant than human and cow. Extensive damage to all vitrified tissues. Equilibrium cooling more appropriate than vitrification.
Ishijima et al, 2006	Dog	1mm^3, 9mm^3, or 125mm^3	DAP213 (4M DMSO, 2M acetamide, 6M Propylene glycol)	Cryotube	Xenotransplantation to NOD-SCID mice, histological analysis, immunohistological analysis (PCNA)	No antral follicle formation after grafting, PCNA detectable in granulosa cells of primary follicles.
Hani et al, 2006	Mouse	1mm^3	DAP213 (4M DMSO, 2M acetamide, 6M Propylene glycol)	Cryotube	Orthotopic transplantation, birth of GFP- positive pups	GFP-positive birth rate highest when ovaries from younger mice were cryopreserved. Follicles of adult mice also retained ability to develop.
Hasegawa et al, 2006	Mouse	Whole ovaries	15% EG, 15% DMSO, 0.5M sucrose, 20% SSS	Polyester sheets	Histological analysis; IVM, IVF, full-term birth of pups – in mice	Oocytes enclosed in preantral follicles from vitrified ovaries retained ability to develop into pups after IVG, IVM and IVF.
Choi et al, 2007	Mouse, rabbit, pig	Whole ovaries (mouse), 5x5x5 mm (rabbit and pig)	40% EG, 10% Ficoll, 0.5M sucrose, 20% FBS	EM Grids	Histological analysis, RT-PCR, caspase-3 assay, Lucifer yellow versus staining,	Cryopreservation inhibits the development of primordial follicles in 1d old mouse ovaries
Isachenko et al, 2007	Human	1x 1-1.5 x 0.7-1 mm	1) 20% EG, 20% DMSO	Cryovial	Histological analysis, hormone assay (E_2 and P_4)	Compared conventional freezing with rapid freezing, concluded that conventional freezing is more promising
Kagawa et al, 2007	Mouse	0.2-0.3mm^3	15% EG, 15% DMSO	Cryotop	In vivo growth, histological analysis, IVM, IVF, ET,	Maturation rate higher, but no difference in cleavage rate between control and vitrified group. 10 pups born from embryo transfer of isolated preantral follicles.
Santos et al, 2007	Goat	1mm^3	40% EG and/or 40% DMSO with/without 0.5M Sucrose	Plastic Straw	Histological analysis, viability (Hoechst, calcein-AM, ethidium homodimer)	Viability of follicles best preserved after SSV in a CP containing sucrose and EG
Aerts et al, 2008	Mouse	< 0.5mm^3	2.8M DMSO, 3.6M EG, 1M Sucrose	SSV	Live-dead fluorescent probes, histological analysis, immunohistological analysis (PCNA)	Primordial follicles decreased and primary follicles increased in vitrified groups as compared to control.
Bagis et al, 2008	Mouse	Whole Ovaries	20% DMSO, 20% EG, 0.5M sucrose	Cryotube	Orthotopic transplantation, litter size, immunohistological analysis (SP2-III, anti-mouse IgG FITC, DAPI), histological analysis	Normal reproductive lifespan can be restored by orthotopic transplantation in transgenic mice. Normal morphology observed in vitrified tissues. Antral follicles present in grafts.
Huang et al, 2008	Human	5x1x1 mm	20% DMSO, 20% EG	SSV	Histological analysis, TUNEL assay, hormone assay (E_2, P_4)	No difference between SSV and slow freezing groups for E_2 and P_4 concentrations or follicle proportions.
Lin et al, 2008	Mouse	Whole ovaries	6M EG, 0.4M Trehalose, 10% FBS	SSV	IVM, IVF after heterotopic transplantation and in vitro culture	Compared droplet size on SSV, no difference between 2µl and 6µl on follicle survival, antral formation, cleavage rate or blastocyst rate
Liu et al, 2008	Mouse	Whole Ovaries	DAP213 (4M DMSO, 2M acetamide, 6M Propylene glycol)	Cryotube	Orthotopic Transplantation, PCR.	Cryopreservation had no effect on long term fertility and reproductive characteristics of recipients

Kagawa et al, 2009	Human, Cow	1x1x10 mm	20% EG, 20% DMSO, 0.5M Sucrose	Metal "Cryotop"	Propidium iodide/Hoechst (viability), histological analysis, immunohistochemistry (PCNA)	High oocyte survival
Keros et al, 2009	Human	1-2 mm^3	1.4M DMSO, 1.5M PROH, 1.5M EG, 10% PVP	0.5ml Cut Plastic Straws	Histological analysis, ultra structural analysis,	Ovarian stroma significantly better preserved after vitrification that after slow freezing
Moniruzzaman et al, 2009	Pig (15 d-old)	2x1x0.5 mm	15% EG, 15% DMSO, 20% FBS, 0, 0.25 or 0.5M sucrose	Cryotop sheet	Histological analysis,	Vitrified tissues maintain ability to develop, developed slower than fresh tissues.
Mazoochi et al, 2009	Mouse	Whole ovaries	40% EG, 30% Ficoll 70, 1M Sucrose	Plastic Straws	Trypan blue, histological analysis, ultra structural analysis, RT-PCR, Flow cytometry	Vitrification affects the expression of some apoptosis-related genes
Zhang et al, 2009	Mouse	1x1x1 mm	1) 20% EG, 20% DMSO, 60μM Z-VAD-FMK, 0.4M sucrose, 15%FBS 2) 20% EG, 0% DMSO, 0.4M sucrose, 15% FBS	0.25ml plastic straw	TUNEL assay, BAX protein detection,	More TUNEL positive follicles in group with caspase inhibitor than without
Zhou et al, 2009	Human	1x1x1 mm	1) 10% EG, 10% DMSO 2) 15% EG, 15% DMSO 3) 20% EG, 20% DMSO 4) 20% EG, 20% DMSO, 0.5M sucrose	Direct LN_2	Histological analysis, TUNEL assay, TEM, heterotopic allograft	Proportion of morphologically normal follicles highest in CP group 2. No difference in TUNEL results among vitrified groups, TEM showed less damage in CP group 2
Silber et al, 2010	Human	0.75-1mm thick	20% EG, 20% DMSO, 0.5M Sucrose	Cryotissue	Return of menstrual cycle after re-implantation, hormone levels (FSH), histological analysis, viability (Hoechst)	Oocyte survival was higher after vitrification (89%) than slow freezing (42%). Normal serum FSH and cyclicity returned by 5 months post-surgery.
Faheem et al, 2011	Cow	10x10x1 mm	15% EG, 15% DMSO	Cryovial	Oocyte aspiration, IVM, IVF.	Higher maturation rate in slow freezing group over vitrification, no difference between cleavage rate and embryonic development rate
Amorim et al, 2012	Human	$1mm^3$	1) 20% DMSO and 20% EG 2) 10% DMSO, 26% EG, 2.5% PVP and 1M Sucrose	Open cryostraws	Histological analysis, TUNEL, Ultra structural analysis, immunohistochemistry (rabbit polyclonal antibody, Ki-67)	Vitrification preservers preantral follicle morphology and survival after 1 week of xenografting and tissues retain ability to resume folliculogenesis
Carvalho et al, 2013	Goat	3x3x1 mm, hemi ovaries, whole ovaries	0.25M sucrose, 20% EG, 20% DMSO	OTC (ovarian tissue cryosystem)	Histological analysis, ultra structural analysis	Decrease in rate of normal preantral follicles as compared to control. Vitrified fragments had higher follicle survival rate than hemi and whole ovaries
Choi et al, 2013	Mouse	Whole ovaries	1) 20% EG, 15% DMSO 2) 40% EG, 18% ficoll, 0.5M Sucrose	Electron microscope grid	RNA extraction, western blot analysis (VEGF, Angpt-2)	Levels of mRNA and protein for VEGF and Angpt-2 significantly reduced in vitrified groups. CP protocol 2 better at preserving angiogenic factors
Ting et al, 2013	Monkey	3x3x0.5 mm	GLY, EG and PVP at varying concentrations	Plastic Straw	Histological analysis, hormone assay (E_2, P_4) BrdU culture	GLY, EG and polymers able to preserve tissue and follicle morphology and function of a small population of secondary follicles

Bandeira et al, 2014	Sheep	3x3x1 mm	1) 2.62M DMSO, 2.60M acetamide, 1.31M propanediol, 0.0075M PEG. 2) 0.25M Sucrose, 20% EG and DMSO.	SSV and OTC	Histological analysis, Ultra structural analysis trypan blue assay, 4Immune-histochemistry (PCNA)	Higher proportion of normal and viable follicles in OTC cultured tissues than SSV.
Fabbri et al, 2014	Human	1.5x0.5x0.2 cm	3M PROH, 5M EG, 0.2M Sucrose	Cryovial	Histological analysis, TUNEL assay, ultra structural analysis	Stromal cells maintained morphology and ultrastructure in vitrified group, oocyte cytoplasm was slightly damaged
Faustino et al 2014	Goat	3x3x1 mm	0.25M Sucrose, 20% EG, 20% DMSO	OTC	Histological analysis, Ultra structural analysis, TUNEL assay, immunofluorescence	Proportion of normal follicles decreased in vitrified/cultured group. DNA damage did not significantly increase after vitrification
Klocke et al, 2014	Human	5x5x1 mm	15% EG, 15% DMSO, 0.5M Sucrose	Direct LN_2	Hormone assay (E_2), Histological analysis, Caspase-3 assay.	Storage at supra-zero conditions has detrimental effects on human ovarian cortex.
Tian et al, 2015	Human	2.5-7 x 1.4-5.2 x 0.4-1.2 mm	2M EG, 2M DMSO, 0.2M Sucrose 2M EG, 2M DMSO, 0.2M Trehalose	Direct LN_2	Histological analysis, stress relaxation test, quasi-linear viscoelasticity	No difference between sucrose and trehalose

Table B: Experimental design for chapter 3: the avian chorioallantoic membrane culture system: a short-term grafting method for bovine ovarian tissue.

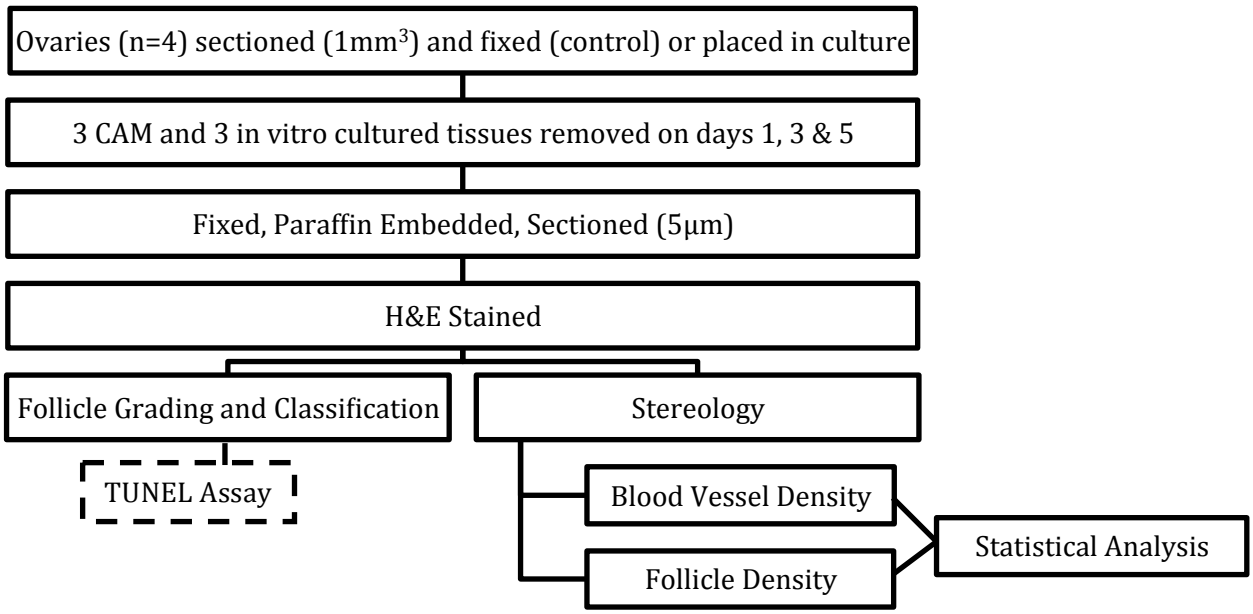

Table C: Total, healthy and degenerated primordial and activated preantral (primary+secondary) densities (number of follicles per mm^3) and proportion of healthy primordial and activated preantral follicles (number over total; %) over 5-day incubation period in the control, in vitro and CAM culture systems

	Control	In Vitro			CAM		
	D0	D1	D3	D5	D1	D3	D5
Total Primordial Follicle Density	40±9.2	15±5.6	14±4.5	11±2.3	30±9.2	34±8.0	19±1.1
Total Activated Preantral Follicle Density	60±20.8	51±26.8	76±41.2	57±15.6	50±18.8	102±31.2	80±20.1
Healthy Primordial Follicle Density	37±8.9	13±5.2	9±4.2	7±2.3	27±7.1	28±7.4	12±1.4
Healthy Activated Preantral Follicle Density	57±19.8	41±21.7	63±34.2	39±12.2	46±19.0	82±31.5	54±17.0
Degenerated Primordial Follicle Density	3±0.6	2±0.9	4±0.8	3±0.1	2±0.5	7±0.7	8±1.7
Degenerated Activated Preantral Follicle Density	3±1.4	10±5.4	13±7.6	18±4.9	4±1.1	20±3.1	26±3.1
Proportion – Healthy Primordial Follicles	39±3.7	25±7.3	18±7.3	11±5.0	38±5.6	22±3.5	13±3.0
Proportion-Healthy Activated Preantral Follicles	55±5.0	56±9.9	55±11.7	55±5.9	52±8.4	51±12.1	49±8.7

Data presented as mean±SE

Table D: Experimental design for chapter 4: the effect of cryoprotectants and cooling devices on the survival of bovine ovarian tissue grafted to the avian chorioallantoic membrane